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Nitroaromatic pro-drug activation and resistance in the African trypanosome

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Table of contents

List of figures

List of tables

List of abbreviations

B-PER™	Bacterial protein extraction reagent
BSF	Bloodstream-form
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
cmc	Critical micellisation concentration
CNS	Central nervous system
CPR	Cytochrome P450 reductase
DDM	n-Dodecyl-β-D-maltoside (synonym: -maltopyranoside)
DFMO	DL-α-difluoromethylornithine (eflornithine)
DHFR-TS	Dihydrofolate reductase-thymidylate synthase
DMPK	Drug metabolism and pharmacokinetics
DND*i*	Drugs for Neglected Diseases *initiative*
DTT	Dithiothreitol
ESI	Electrospray ionisation
FRase	Flavin reductase (from *Vibrio fischeri*)
FxR	Fexinidazole-resistant
gDNA	Genomic deoxyribonucleic acid
HAT	Human African trypanosomiasis
HRP	Horseradish peroxidase
HYG	Hygromycin phosphotransferase
IBSR	Inclusion body solubilisation reagent
IMAC	Immobilised metal affinity chromatography
i.m.	Intramuscular(ly)
i.p.	Intraperitoneal(ly)
IPTG	Isopropyl-β-D-thiogalactopyranoside
i.v.	Intravenous(ly)

LB	Luria-Bertani
LC	Liquid chromatography
MW	Molecular weight
MWCO	Molecular weight cut-off
NECT	Nifurtimox-eflornithine combination therapy
NEO	Neomycin phosphotransferase
NfxR	Nifurtimox-resistant
NOX	NADH-oxidase (from *Thermus thermophilus*)
NTA	Nitrilotriacetic acid
NTR	Nitroreductase
NusA	N-utilising substance A
OD_{600}	Optical density at $\lambda = 600$ nm
ORF	Open reading frame
OYE	Old yellow enzyme
PAC	Puromycin acetyltransferase
PCI	Phenol/chloroform/isoamyl alcohol
PGS	Prostaglandin synthase
RP	Reverse phase
RT	Room temperature
SAP	Shrimp alkaline phosphatase
S.D.	Standard deviation
S.E.M.	Standard error of the mean
SKO	Single knock-out
SNP	Single nucleotide polymorphism
T7RPOL	Bacteriophage T7 RNA polymerase
TETR	Tet repressor

TEV	Tobacco etch virus protease
Ti	Tetracycline inducible
TryP	Tryparedoxin peroxidise
TryR	Trypanothione reductase
TryS	Trypanothione synthetase
Tsf	Elongation factor Ts
UPLC	Ultraperformance liquid chromatography
UTR	Untranslated region
VSG	Variant surface glycoprotein

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Declaration

I hereby certify that this thesis is of my own composition and is based upon the results of my own work, carried under the supervision of Professor Alan H. Fairlamb in the College of Life Sciences, University of Dundee. Work other than my own is clearly specified in the text by reference to the relevant researchers or to their publications. I have consulted all references cited herein, unless otherwise stated. No part of this thesis has been previously submitted for a higher degree.

Antoaneta Y. Sokolova

I certify that Antoaneta Sokolova has performed the research described in this thesis under my supervision and has fulfilled the conditions of the relevant ordinance and regulations of the University of Dundee, and that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

Professor Alan H. Fairlamb

Wellcome Principal Research Fellow

Abstract

Sleeping sickness, caused by *Trypanosoma brucei*, is a deadly disease that affects some of the poorest countries in sub-Saharan Africa. Although the disease prevalence is declining, strengthening of the current control efforts, including introduction of more adequate chemotherapeutic options, is needed to prevent the re-emergence of yet another epidemic. Nitroaromatic compounds, such as nifurtimox (in combination with eflornithine) and fexinidazole (in clinical trials), have been recently introduced for the treatment of the second stage of sleeping sickness. These compounds are believed to act as pro-drugs that require intracellular enzymatic activation for antimicrobial activity. Here, the role of the bacterial-like nitroreductase *Tb*NTR as a nitrodrug activating enzyme is examined through overexpression and knock-out studies in *T. brucei*. Multiple attempts to purify soluble recombinant *Tb*NTR from *E. coli* were unsuccessful, because the recombinant protein was found to be membrane associated. In keeping with the role of *Tb*NTR in nitrodrug activation, loss of an *NTR* gene copy in *T. brucei* was found to be one, but not the only, mechanism that may lead to nitrodrug resistance. Furthermore, in the bloodstream form of *T. brucei*, resistance was relatively easy to select for nifurtimox, with no concurrent loss of virulence and at clinically relevant levels. More worryingly, nifurtimox resistance led to a decreased sensitivity of these parasites to other nitroaromatic compounds, including a high level of cross-resistance to fexinidazole. Conversely, generation of fexinidazole resistance resulted in cross-resistance to nifurtimox. Should these findings translate to the field, emerging nitrodrug resistance could reverse all recent advances in the treatment of sleeping sickness, made since the introduction of eflornithine 20 years ago. Therefore, all efforts should be made to ensure nitroaromatic drugs are used only in drug combination therapies against sleeping sickness, in order to protect them from emerging resistance.

Chapter 1 -

Introduction

1.1. Human African trypanosomiasis and the African trypanosome

Human African trypanosomiasis (HAT, sleeping sickness) is one of the deadliest neglected tropical diseases, which is considered invariably fatal if untreated. This vector-borne parasitic disease is endemic in 36 sub-Saharan African countries, placing a population of ~60 million people at risk. Yet, fewer than 6 million people have been under active surveillance at any one time. As a result, during the peak (1997 – 1998) of the latest sleeping sickness outbreak, fewer than 40,000 cases were reported per year, whereas the annual total number of cases was estimated to be in the range of 300,000 to 500,000 (Brun *et al*., 2010; Simarro *et al*., 2008; Stuart *et al*., 2008; WHO Expert Committee, 1998). The prevalence of HAT has subsequently been reduced (**Figure 1.1**) through the implementation of vector control and population surveillance programmes, as well as through improving awareness and access to diagnostic and treatment tools in the disease-endemic countries. Currently, the number of reported HAT cases is at its lowest level for half a century (<10,000 cases in 2009), and the estimated number of total cases is only 30,000 per year (Human African trypanosomiasis (sleeping sickness): epidemiological update, 2006; Simarro *et al*., 2011). However, this temporary success might affect negatively the maintenance of control and surveillance activities (Simarro *et al*., 2008). Cessation of such activities in the 1960s, combined with population displacement and exposure to hidden disease foci, was thought to be the main trigger for resurgence of the disease. Although in the past, this was primarily caused by civil unrest and lack of political stability, neglect of control activities due to any circumstances could easily lead to yet another sleeping sickness epidemic in the future (Simarro *et al*., 2011; Stuart *et al*., 2008).

1.1.1. *Trypanosoma brucei* – the aetiological agent

African trypanosomiasis is caused by the obligate parasite *Trypanosoma brucei*, of the order Kinetoplastida. Species of this order are unicellular, motile, flagellated protozoa, characterised by the presence of a single large mitochondrion, which contains a compacted DNA network termed the "kinetoplast" (Stuart *et al*., 2008). There are three subspecies of *T. brucei*, two of which – *T. b. gambiense* and *T. b. rhodesiense*, are the aetiological agents of the human form of the disease known as sleeping sickness. The third subspecies, *T. b. brucei*, is non-infective to humans but causes a similar wasting disease in cattle (nagana, or "depressed and low in spirits"; Fairlamb, 2003).

The three subspecies of *T. brucei* are morphologically indistinguishable from each other but differ in their geographical distribution (**Figure 1.1** and section 1.1.2), host specificity, and in the case of the human-infective forms, in disease progression. *T. b. gambiense*, which accounts for the majority (~95%) of HAT cases, is endemic in foci throughout West and Central Africa. Humans are thought to be the main reservoir for *T. b. gambiense*, and only few other animals have been found to carry this subspecies of *T. brucei* (Burri and Brun, 2009). In terms of disease progression, *T. b. gambiense* causes the more chronic form of sleeping sickness, which on average takes 2 – 3 years from onset to death in untreated patients, but on rare occasions, it can last over 6 – 7 years (Checchi *et al*., 2008a; Checchi *et al*., 2008b). By contrast, *T. b. rhodesiense* is prevalent in the eastern parts of Africa and causes the more acute form of sleeping sickness, which progresses from infection to death within 3 – 12 months (Odiit *et al*., 1997, as cited by Fevre *et al*., 2004). However, sleeping sickness due to *T. b. rhodesiense* is rare (<5% of all HAT cases; Simarro *et al*., 2011) and is generally considered a zoonosis. In addition to humans, *T. b. rhodesiense* can infect many wild and domestic animals, of which cattle are an important reservoir (Burri and Brun, 2009). The last subspecies, *T. b. brucei* is spread throughout sub-Saharan Africa and is

closely related to *T. b. rhodesiense* (Balmer *et al*., 2011). *T. b. brucei* also affects a wide range of vertebrates but is not pathogenic to humans, due to its susceptibility to lysis by human serum (Vanhamme and Pays, 2004).

1.1.2. Transmission and life cycle of *T. brucei*

African trypanosomiasis is a vector-borne disease, transmitted by species of the genus *Glossina* (order Diptera), commonly known as tsetse flies. There are over 20 *Glossina* spp., all of which are restricted by climatic and environmental factors to the tropical and sub-tropical parts of Africa, between the Sahara and Somali deserts to the north and the Kalahari and Namibian deserts to the south (Jordan, 1993). Within these broad geographical limits, specific habitat requirements provide the basis for separation of the *Glossina* genus into three groups. Species of the *fusca* group, which are forest dwellers, do not commonly feed on humans and have not been shown to act as vectors of human-infective *T. brucei*. By contrast, species of the *morsitans* group, which are responsible for the transmission of *T. b. rhodesiense*, are found in foci throughout the savannah woodlands of East Africa (**Figure 1.1**). Finally, species of the *palpalis* group are found along river banks and lake shores, as well as in the lowland rainforests of West and Central Africa, where they transmit *T. b. gambiense* (Jordan, 1993). An important exception is the transmission of *T. b. rhodesiense* by species of the *palpalis* group in south-east Uganda. Uganda is the only country in which both forms of sleeping sickness exist: *T. b. gambiense* to the north-west and *T. b. rhodesiense* to the south-east (Human African trypanosomiasis (sleeping sickness): epidemiological update, 2006). Although these two disease foci are currently isolated (Batchelor *et al*., 2009; Picozzi *et al*., 2005), a potential overlap between them will present huge difficulties in terms of the correct choice of diagnostic and treatment tools, which differ substantially between the two forms of the disease (see sections 1.1.3 and 1.2).

The restrictive habitat requirements of *Glossina* spp., combined with their dependence on the presence of woody vegetation for resting sites (tsetse flies rest for >23 h per day; Jordan, 1993), account for the fact that transmission of sleeping sickness occurs predominantly in rural settings. Thus, the communities affected by African trypanosomiasis often live in remote and very poor areas, which have little or no access to healthcare facilities and are difficult to reach by trypanosomiasis control and surveillance teams (Brun *et al*., 2010; Chappuis *et al*., 2010; Fevre *et al*., 2006). Such a lack of healthcare and infrastructure has implications not only on the patients, many of which remain undiagnosed and do not receive treatment, but also on the tools that can be used to fight sleeping sickness. In addition to being safe and effective, these tools also need to be cost efficient, easy to use/administer in the field, stable under tropical conditions and ideally not require the use of elaborate equipment (Deborggraeve and Buscher, 2010; Frearson *et al*., 2007; Simarro *et al*., 2008).

Transmission of *T. brucei* is biological, which means that trypanosomes must undergo an essential part of their life cycle in the insect vector (**Figure 1.2**), before they can become infective to the mammalian host (Medical Insects and Arachnids, 1993; Fenn and Matthews, 2007). Mechanical transmission of *T. brucei*, whereby no development takes place in the vector, has been demonstrated only under laboratory conditions and is highly unlikely to play any role in the field (as discussed by Jordan, 1993 and Moloo *et al*., 2000). In tsetse flies, the development of *T. brucei* takes 3 – 5 weeks (Brun *et al*., 2010), during which trypanosomes go through a series of proliferative and non-proliferative (adaptive) life cycle stages, whilst migrating from the midgut of the fly to the salivary glands (**Figure 1.2**; Vickerman, 1985). Completion of this process is not always successful and depends on many vector- and parasite-specific factors (Matthews, 2011; Welburn and Maudlin, 1999). As a result, only ~0.1% of tsetse flies are found to carry matured human-infective trypanosomes in the field. However,

once an infection has been established, a fly can potentially transmit the disease to all suitable hosts it feeds on for the rest of its life* (Jordan, 1993; Stuart *et al*., 2008).

In humans, the life cycle of *T. brucei* begins when an infected tsetse fly deposits mature metacyclic trypanosomes in the dermal connective tissue during a blood meal (Vickerman, 1985; **Figure 1.2**). At the bite site, a localised inflammatory skin response may lead to the formation of a painful trypanosomal chancre (a hard, defined swelling; Burri and Brun, 2009), where the infective, non-dividing metacyclics transform into proliferative slender bloodstream forms (BSF). The latter escape the chancre through the draining lymphatics, and migrate to the lymph nodes and eventually, to the bloodstream. During the first stage of sleeping sickness, slender forms multiply extracellularly in the haemolymphatic system, at 5- to 10-hourly intervals (Fries and Fairlamb, 2003; Vickerman, 1985). Throughout the infection, the levels of parasitaemia in the blood fluctuate in a wave-like pattern (first described by Ross and Thomson, 1910). The main reason for these fluctuations is thought to be an arms-race between the parasite, which constantly changes the composition of its surface coat by antigenic variation, and the immune system of the host, which responds by generating antibodies against the main circulating variant of *T. brucei* (Pays and Nolan, 1998). In addition to the immune system, trypanosomes may further be responsible for the successive declines in parasitaemia, by differentiating into non-dividing, stumpy forms at high densities (**Figure 1.2**). These forms, unlike slender BSF trypanosomes, are believed to be pre-adapted for survival in the insect vector, where they continue to differentiate, in order to complete their life cycle (Fenn and Matthews, 2007). With the progression of the disease, trypanosomes cross the walls of the blood vessels and the choroid plexus to

* It is difficult to estimate the epidemiological impact of a single infected fly, due to conflicting reports on the longevity of *Glossina*, the role of a *T. brucei* infection thereon, and the number and frequency of blood meals. Further difficulties are presented by the specific host preferences, which vary hugely among *Glossina* spp., and the availability of hosts the fly can feed on, not all of which may be susceptible to the particular subspecies of *T. brucei* (Fevre *et al*., 2006; Jordan, 1993; Pepin and Meda, 2001; Welburn and Maudlin, 1999; WHO Expert Committee, 1998).

invade the central nervous system (CNS), which marks the beginning of the second, final stage of HAT (Fries and Fairlamb, 2003). To date, it has not been established with certainty whether trypanosomes differentiate into new forms during CNS invasion, or whether they remain morphologically and biochemically identical to those found in the bloodstream. Early studies with laboratory rodents report a variety of morphological *T. brucei* forms in the brain, including intracellular trypomastigotes (Abolarin *et al*., 1982) and even amastigotes (Beckers *et al*., 1981). More recently, it has been speculated that trypanosomes could enter the CNS by migrating through the cells of the blood-brain barrier (thus presenting as intracellular trypomastigotes) rather than through the tight junctions between these cells (Enanga *et al*., 2002; Stoppini *et al*., 2000). The morphology of *T. brucei* in the brain is perhaps less important than the biochemical composition of these parasites, which has yet to be characterised. If CNS trypanosomes are biochemically different from their bloodstream counterparts, it is possible that they might respond differently to chemotherapy (Enanga *et al*., 2002). Last but not least, the ability of trypanosomes to proliferate in the CNS has not been directly demonstrated (Enanga *et al*., 2002), although some authors seem convinced that *T. brucei* do multiply by binary fission not only in the blood and lymph but also in the CNS (Vickerman, 1985). What has been demonstrated on multiple occasions, however, is that trypanosomes within the brain can re-establish an infection in the bloodstream. For example, following an apparent clearance of *T. brucei* from the blood of infected mice, brain homogenates (but not blood) from these mice could cause an infection in recipient animals (Gray *et al*., 1982). In a similar way, *T. b. gambiense* isolates from the cerebrospinal fluid of second-stage HAT patients were able to establish an infection in laboratory animals and to a lesser extent to proliferate *in vitro* (Giroud *et al*., 2009). If trypanosomes are not completely cleared by chemotherapy, not only can they infect other organisms/recipients, but also re-establish an infection in the same individual, as

has been demonstrated in a melarsoprol-refractory *T. b. rhodesiense* patient (Checkley *et al*., 2007). Collectively, these studies indicate that should drug resistance be selected in the course of chemotherapy, *T. brucei* have the ability to re-emerge in the bloodstream, where they would not only cause a relapse in the infected individual, but also be available for transmission by the blood-feeding vector.

The successful transmission of *T. brucei* is partly dependent on their ability to survive the transition from host to vector and *vice versa*. To adapt to these very different environments, trypanosomes undergo complex morphological and biochemical changes during the various stages of their life cycle. Some of the most prominent of these changes occur in the mitochondrion, regarding utilisation of the available energy sources (Vickerman, 1985). In the midgut of the fly, where glucose is present only transiently after a blood meal, procyclics are thought to use proline – similar to their vector, and potentially several other amino acids, as their main source of energy. The mitochondrion of procyclic *T. brucei* is relatively enlarged, with its branched inner membrane forming discoid cristae and featuring a fully functional, cytochrome-dependent electron transport chain, which generates a proton gradient to drive the synthesis of ATP (Besteiro *et al*., 2005; Vickerman, 1985). By contrast, in the mammalian bloodstream, where glucose is constantly available, slender BSF trypanosomes dispense with the components of the cytochrome chain and the tricarboxylic acid cycle, and generate ATP solely and wastefully by aerobic glycolysis. In addition, the smaller and smoother, cristae-lacking mitochondrion of BSF *T. brucei* functions at a reduced capacity, consuming rather than generating ATP in order to maintain the proton gradient across its membranes. Nonetheless, in slender forms, the mitochondrion remains an essential organelle, due to its role in maintaining the glycolytic redox balance (Besteiro *et al*., 2005; Vickerman, 1965; Vickerman, 1985). Another set of important changes that occur as trypanosomes transit from host to vector

and *vice versa* takes place on the cell surface, which is completely covered by a monolayer of densely packed, stage-specific glycoproteins (Pays and Nolan, 1998). In procyclic *T. brucei*, this surface coat is composed of procyclic acidic repetitive proteins (procyclins), which are thought to be highly resistant to the proteases of the tsetse midgut. Furthermore, the large carbohydrate chains covalently attached to procyclins are thought to form a glycocalyx to protect the remaining surface proteins (Mehlert *et al*., 1998; Pays and Nolan, 1998). Although in the mammalian bloodstream *T. brucei* are not threatened by such digestive enzymes, they are constantly exposed to the immune system of the host, from which they are protected by a coat of variant surface glycoproteins (VSG). The VSG monolayer is sufficiently dense to restrict access to the plasma membrane for components of the complement pathway of the innate immune system, but not for small molecules and nutrients (Mehlert *et al*., 1998). To evade the acquired immune system, BSF *T. brucei* periodically change the composition of their surface coat to a new antigenic variant of VSG, thereby avoiding recognition by antibodies against previous variants (Pays, 2006). Although in the *T. brucei* genome there are over 1000 *VSG* genes, only one of which is expressed at any given time, the true repertoire of antigenic variants is virtually limitless due to the ability of trypanosomes to modify the existing *VSG* sequences by homologous DNA recombination (Pays, 2006; Stuart *et al*., 2008). On the one hand, this constant variation of the antibody-accessible coat ensures the survival of at least a small proportion of the trypanosome population by preventing the host from effectively clearing the infection without treatment. On the other hand, the inexhaustible repertoire of antigenic variants precludes the development of a potential vaccine against HAT, which leaves chemotherapy and vector control as the only options for the millions under threat of contracting the disease (Fries and Fairlamb, 2003; Stuart *et al*., 2008).

1.1.3. Clinical manifestation and diagnosis of HAT

Sleeping sickness caused by either *T. b. gambiense* or *T. b. rhodesiense* progresses in two stages and is ultimately fatal if untreated. However, the clinical features and duration, as well as the available diagnostic tools, differ between the two forms of the disease (Brun *et al*., 2010). During the first, haemolymphatic stage of the more chronic *T. b. gambiense* infection, symptoms vary between patients, and are highly unspecific and reminiscent of other microbial and viral infections, including malaria. These symptoms often include fever, headache, lymphadenopathy, joint and muscle pain, fatigue and general malaise, all of which occur in cycles as the immune system responds to the successive waves of parasitaemia. More specific symptoms of stage-one chronic sleeping sickness include pruritus and facial oedema, as well as the classic Winterbottom's sign, which is manifested as a swelling of the posterior cervical lymph nodes in approximately 85% of clinical patients (Brun *et al*., 2010; Burri and Brun, 2009; Stuart *et al*., 2008). With time, the febrile episodes appear with reduced frequency and intensity, and are usually followed by a long asymptomatic period (Burri and Brun, 2009). By contrast, stage-one sleeping sickness due to *T. b. rhodesiense* progresses much more acutely, and fever develops within a few weeks of infection (Burri and Brun, 2009). A trypanosomal chancre, not normally observed in *T. b. gambiense* patients, may also appear in the first 1 – 2 weeks at the site of the infective tsetse bite. Conversely, the Winterbottom's sign, which is characteristic of West African trypanosomiasis, is usually absent in *T. b. rhodesiense* patients, although other lymph nodes (submandibular, axillary and inguinal) may be affected during this stage (Brun *et al*., 2010; Burri and Brun, 2009).

The leading symptoms of second-stage *T. b. gambiense* infections are predominantly neurological, due to the invasion of the CNS by trypanosomes and the resulting chronic inflammation of the brain. These symptoms can be classified as motor

(slurred speech, tremors), sensory, sleep and mental anomalies, the latter, ranging from highly aggressive, manic behaviour to confusion and complete apathy. Sleep anomalies, after which the disease was named, are also highly characteristic of the second stage of West African trypanosomiasis and include dysregulation of the sleep-wake cycle and fragmentation of the sleep pattern (Brun *et al*., 2010; Burri and Brun, 2009; Fries and Fairlamb, 2003). Other symptoms, which are of minor clinical relevance in *T. b. gambiense* infections, such as cardiac and endocrine dysfunctions, may become more pronounced in second-stage *T. b. rhodesiense* patients. Furthermore, many of the neurological symptoms are absent in the East African form of sleeping sickness, probably due to the much faster progression of the disease (Brun *et al*., 2010; Burri and Brun, 2009). Although most of the brain damage that occurs during chronic sleeping sickness may be reversed following treatment, in the absence of treatment, patients will continue to deteriorate (Burri and Brun, 2009; Fries and Fairlamb, 2003). Unlike stage-one symptoms, the symptoms of second-stage chronic sleeping sickness increase in frequency and severity with time, eventually leading to considerable weight loss, coma and death, often caused by concurrent bacterial infections. Untreated *T. b. rhodesiense* sleeping sickness will also lead to death, but at a much faster rate (Brun *et al*., 2010).

The clinical features of sleeping sickness are not sufficiently specific to exclude the presence of other brain or chronic inflammatory conditions. Therefore, before the patients are exposed to the highly toxic drugs that are currently available for the treatment of HAT (see section 1.2), confirmation of the disease by laboratory tests is required (Burri and Brun, 2009; Chappuis *et al*., 2005). In *T. b. gambiense*-endemic countries, diagnosis of HAT usually follows a three-step process of screening, diagnostic confirmation and staging. Initial screening of large number of patients in the field is performed with the relatively quick and easy-to-use card agglutination test for trypanosomiasis (CATT/*T. b. gambiense*; developed by Magnus *et al*., 1978), which

uses a single, frequently-encountered variable antigen type of *T. b. gambiense* to detect antibodies in the patient's blood. However, this test is limited in both its sensitivity (may not detect patients with *T. b. gambiense* of other antigen types) and specificity (may detect patients with malaria or with transient non-infective trypanosomes; Chappuis *et al*., 2005). Although other more sensitive serological tests, such as immunofluorescent and enzyme-linked immunosorbent (ELISA) assays, are also available, these remain confined to laboratory settings by the need for elaborate equipment. Regardless of their sensitivity or specificity, serological tests are not able to distinguish recent from current infections, as antibodies remain in circulation for years after the infection has been cleared (Chappuis *et al*., 2005). Hence, to confirm the diagnosis of seropositive individuals, detection of the trypanosomes by parasitological or molecular methods is required. Under field conditions, the method of choice for parasitological confirmation is microscopy on lymph node aspirates or blood samples, despite the unsatisfactory detection limit (5,000 – 10,000 trypanosomes ml^{-1}). Where appropriate facilities exist, this limit could be reduced to 100 – 500 trypanosomes ml^{-1} by prior concentration of the sample using field-adapted centrifugation techniques. Nevertheless, during this process, as many as 30% of infected patients may remain undiagnosed, due to the highly variable levels of parasitaemia, which can fall below 100 *T. b. gambiense* cells ml^{-1} (Chappuis *et al*., 2005). The alternative parasitological methods, whereby potentially infected blood is used to inoculated suitable culture media or susceptible laboratory animals, are unsuitable for diagnostic purposes because viable trypanosomes can only be detected after several days or even weeks post inoculation (Burri and Brun, 2009; Chappuis *et al*., 2005). In addition, there are several extremely sensitive, molecular-based techniques (summarised in Deborggraeve and Buscher, 2010) for the detection of trypanosomes, such as DNA/RNA amplification or fluorescence *in situ* hybridisation. However, these complex, molecular-based techniques

remain currently inapplicable in the resource-poor setting of rural Africa, despite various strategies that have been undertaken to simplify them. Furthermore, the proper evaluation of these methods on a large scale, as well as the clinical significance of detecting very low levels of trypanosomal DNA/RNA in patients, have been questioned (Chappuis *et al*., 2005; Deborggraeve and Buscher, 2010). Finally, the last diagnostic step for patients with confirmed sleeping sickness is determination of the disease stage. At present, second-stage HAT can be detected solely by examination of the cerebrospinal fluid for an increased white blood cell count (variable lower limit of 5 – 20 cells μl^{-1}) and potentially, for the presence of trypanosomes. Both of these criteria require the use of a microscope and hence, suffer from the same lack of sensitivity as other microscopic methods described above. Another highly specific marker for second stage sleeping sickness is increased levels of IgM in the cerebrospinal fluid, which can be detected by a simple latex agglutination test. However, this method is still awaiting validation and similar to the other criteria, requires the highly invasive lumbar puncture to obtain cerebrospinal fluid (Brun *et al*., 2010; Stuart *et al*., 2008).

The diagnosis of sleeping sickness due to *T. b. rhodesiense* is different from the screening, confirmation and staging pattern established for diagnosis of *T. b. gambiense* infections. To date, no equivalent of any serological tests is available for the screening of human East African trypanosomiasis, although some of the molecular screening methods, based on amplification of subspecies-specific DNA, have been applied to detect *T. b. rhodesiense* in animals (Stuart *et al*., 2008; Welburn *et al*., 2001). Consequently, the initial, speculative diagnosis of *T. b. rhodesiense* infections is based on clinical presentation, which is usually more acute, and on the history of patient exposure to the vector (Brun *et al*., 2010). However, the lack of specificity of this approach has lead to a substantial delay (60 days on average in Uganda) before an accurate diagnosis can be reached and treatment initiated (Odiit *et al*., 2004, as

referenced by Fevre *et al*., 2006). In contrast to *T. b. gambiense* cases, where initial screening is straightforward but subsequent confirmation can be problematic, it is relatively easy to confirm suspected *T. b. rhodesiense* cases by microscopy of blood samples or chancre aspirates, even without prior concentration of the sample, because of the relatively high levels of parasitaemia. Although inapplicable as a diagnostic tool, detection of *T. b. rhodesiense* may also be performed by inoculation into a wide range of laboratory animals, whereas very few, mostly immunocompromised, rodents are susceptible to *T. b. gambiense*. The final, staging part of the diagnostic process is the only one that is identical for *T. b. gambiense* and *T. b. rhodesiense* infections, and carries the same risks for the patient (Burri and Brun, 2009; Chappuis *et al*., 2005). Nonetheless, determination of the stage of sleeping sickness is vital for both *T. b. gambiense* and *T. b. rhodesiense* patients because the treatment options differ considerably between the first and second stage of the disease (Stuart *et al*., 2008).

1.2. Chemotherapy of sleeping sickness

There are five different drugs currently available for the chemotherapy of sleeping sickness (**Table 1.1**). However, not all of them are equally applicable against all forms of the disease. Choice of the correct treatment is highly dependent on the disease stage and the causative subspecies of *T. brucei*, as well as on the availability of the drugs, which can be a major issue in HAT-affected areas (Simarro *et al*., 2011; Stuart *et al*., 2008). Two of the drugs – pentamidine and suramin are effective only against the first, haemolymphatic stage of sleeping sickness, because their physicochemical properties prevent them from penetrating through the blood-brain barrier and into the CNS (Fries and Fairlamb, 2003). The remaining treatment options are effective against both first- and second-stage HAT, although there are other reasons why their use is avoided in stage-one patients, as detailed below.

1.2.1. Drugs against first-stage HAT

Pentamidine

Pentamidine (Pentam 300; Nebupent; Pentacarinat; Lomidine) has been the drug of choice against early-stage *T. b. gambiense* infections since it was first introduced in 1941, because it is relatively well tolerated compared to the remaining HAT treatment options. However, due to its much lower efficacy against *T. b. rhodesiense*, pentamidine is not used against East African trypanosomiasis (Burri and Brun, 2009; Fairlamb, 2003). Pentamidine is an aromatic diamidine, which is highly soluble in water (as the di-isethionate or di-mesylate salt) and almost fully protonated at physiological pH. These properties account for the inability of the drug to cross the blood-brain barrier, as well as for its poor absorption in the gastrointestinal tract, which in turn necessitates parenteral administration (Fries and Fairlamb, 2003). Pentamidine is usually given via a deep intramuscular (i.m.) injection, although this can be painful and in some cases, may lead to sterile abscesses or necroses at the injection site. Nonetheless, this route is preferred over intravenous (i.v.) administration of the drug, because the latter may cause severe and life-threatening hypotension. Additional frequently-observed side effects of this drug include hypoglycaemia (and potentially, diabetes mellitus), gastrointestinal disturbances, blood dyscrasias, and damage to the kidneys, heart and liver (Burri and Brun, 2009; Fries and Fairlamb, 2003).

Suramin

Suramin (Bayer 205; Germanin) is active against first-stage sleeping sickness caused by *T. b. gambiense* and by *T. b. rhodesiense*, although its use against early-stage *T. b. gambiense* infections is not recommended, because of the severe allergic reactions that may develop in patients co-infected with onchocerciasis (common in western and central Africa; Brun *et al*., 2010). Suramin was developed in the early 1920s as part of

the very first two series of synthetic antitrypanosomal agents – synthetic dyes and organic arsenical (Williamson, 1970). Suramin is a dye-related, polyanionic sulfonated naphthylamine with a high molecular mass and polarity, which prevents it from entering the CNS. Similar to pentamidine, suramin is highly water-soluble and requires parenteral administration, although in this case the i.v. route is preferred (Fries and Fairlamb, 2003). During administration of suramin, adverse hypersensitivity reactions may develop, such as nausea and vomiting, pruritus, urticaria and even shock, hence the requirement for a test dose. Other, delayed side effects of suramin include kidney damage (usually reversible), exfoliative dermatitis, peripheral neuropathy, par- and hyperaesthesia of hands and feet, haemolytic anaemia and other blood dyscrasias resulting from bone marrow toxicity (Brun *et al*., 2010; Burri and Brun, 2009; Fries and Fairlamb, 2003).

1.2.2. Drugs against second-stage HAT

Melarsoprol

Since its introduction in 1949, melarsoprol (Mel B; Arsobal) has been the only drug that is active against both *T. b. gambiense* and *T. b. rhodesiense* in the late stages of sleeping sickness (**Table 1.1**; Fries and Fairlamb, 2003). However, melarsoprol is not completely effective. Treatment failure rates, normally in the range of 5 – 8%, soared up to 16 – 30% at the turn of the 21st century in *T. b. gambiense* foci in some of the most affected countries – Angola, Democratic Republic of Congo, Sudan and Uganda (**Figure 1.1 A**). This increase in relapse rates was probably due to emerging drug resistance, although this has not been verified for *T. b. gambiense*, and other vector- and parasite-related factors cannot be excluded (Brun *et al*., 2001; Robays *et al*., 2008). Nonetheless, melarsoprol remains a first-line treatment for late-stage *T. b. gambiense* HAT in many regions which have no access to or cannot afford the use of eflornithine. Melarsoprol is

also the only, hence first-line treatment for second-stage *T. b. rhodesiense* infections, with no effective alternatives available for relapse cases (Simarro *et al*., 2011; Stuart *et al*., 2008). In addition to the failing efficacy, another major disadvantage of melarsoprol is the unacceptable drug toxicity. Melarsoprol is an organoarsenic compound, which triggers severe reactive encephalopathy in 5 – 10% of all treated cases, approximately half of these resulting in fatality. The cause of this extreme adverse reaction is debatable, although it is most probably independent of the trypanosome, as the same reaction has been observed in melarsoprol-treated patients with advanced leukaemia (Fairlamb, 2003; Soignet *et al*., 1999). Unfortunately, an abridged 10-day treatment schedule, which is now recommended for melarsoprol against *T. b. gambiense* (see **Table 1.1**), has failed to reduce the incidence of reactive encephalopathy compared to a standard 26-day regimen. Nonetheless, the shorter regimen offers other important advantages over previous schedules, such as reduction in the hospitalisation time and treatment cost (Burri and Brun, 2009; Fairlamb, 2003; Schmid *et al*., 2004). In all cases, melarsoprol, which is insoluble in water, needs to be administered strictly via an i.v. injection, dissolved in propylene glycol. This solvent is highly irritating and may cause thrombophlebitis in the surrounding tissues if leaked during injection. Administration of melarsoprol is also associated with some common side effects, such as fever, headache, vomiting and abdominal pain, as well as with more severe adverse reactions, including skin irritations (pruritus and maculopapular eruptions), peripheral neuropathy (motor and sensory), myocardial damage, renal dysfunction and hepatotoxicity (Burri and Brun, 2009; Fairlamb, 2003; Fries and Fairlamb, 2003).

Eflornithine

Eflornithine (DL-α-difluoromethylornithine, DFMO; Ornidyl) was recognised as an antitrypanosomal agent in the 1980s; however, it was not until 1990 before it was

approved for the treatment of *T. b. gambiense* cases in both stages of the disease. The drug cannot be used against *T. b. rhodesiense*, which is naturally refractory to eflornithine by virtue of its higher turnover rate of ornithine decarboxylase (the essential enzyme that is inhibited by eflornithine), and some other contributing factors (Fries and Fairlamb, 2003). Even though eflornithine is a much safer alternative to melarsoprol, its high cost ($710 per treatment), logistically-challenging packaging (40 kg per kit containing equipment for 2 full treatments) and complex regimen (56 infusions at 6-h intervals, see **Table 1.1**) have severely limited its use in many remote regions in rural Africa (Simarro *et al*., 2011; Stuart *et al*., 2008). Furthermore, the development of eflornithine as an oral formulation, which was expected to alleviate some of the above issues, has been hampered by the poor and stereoselective absorption of the drug (favouring the less active isomer) following oral dosing (Brun *et al*., 2010; Jansson *et al*., 2008). At present, the main risks for patients receiving i.v. eflornithine is the development of a bacterial infection (potentially leading to sepsis) at the site of injection, although this can be avoided by preventive care. The main adverse reactions associated with administration of eflornithine are bone marrow toxicity with blood dyscrasias, gastrointestinal disturbances (nausea, vomiting and diarrhoea) and convulsions. These effects are caused by the cytostatic action of the drug and are reversible upon cessation of treatment (Burri and Brun, 2009).

Nifurtimox-eflornithine combination therapy (NECT)

The combination of eflornithine with the nitrofuran nifurtimox (Bayer 2502; Lampit) was first evaluated for the treatment of late-stage *T. b. gambiense* in 2001 as part of a clinical trial comparing three drug combinations: melarsoprol with eflornithine, melarsoprol with nifurtimox and eflornithine with nifurtimox. The study was aimed at addressing the multitude of disadvantages of the existing antitrypanosomal drugs, with

the rationale that using these drugs in combination would reduce the possibility of developing drug resistance and would allow lower drug doses to be used, thus potentially reducing toxicity and simplifying drug administration without compromising treatment efficacy. Contrary to these expectations, the clinical trial had to be interrupted on ethical grounds, due to the notably high drug-related mortality in the arms containing melarsoprol (Priotto *et al*., 2006). Nonetheless, the combination of eflornithine with nifurtimox appeared to be relatively effective and well tolerated, which was subsequently confirmed in a randomised phase III clinical trial – the cure rate for NECT was high (96.5%), with fewer patients suffering adverse effects than with eflornithine monotherapy (Priotto *et al*., 2009).

Prior to these clinical trials, nifurtimox had been used mainly to treat acute South-American trypanosomiasis (Chagas' disease; caused by *Trypanosoma cruzi*; Rassi *et al*., 2010), but was never approved as a monotherapy for the treatment of HAT due to its insufficient efficacy (30 – 80% cure rate with different regimens) and dose-dependent toxicity. However, nifurtimox had been used on compassionate grounds in melarsoprol-refractory *T. b. gambiense* patients with no access to eflornithine (Bouteille *et al*., 2003; Pépin and Milord, 1994).

A major advantage of nifurtimox over the existing antitrypanosomal agents is that it can be administered orally. Furthermore, NECT uses half the amount of eflornithine compared to the monotherapy (**Table 1.1**). In turn, the reduced drug quantity helps to alleviate the two main obstacles for the implementation of eflornithine against HAT – cost ($360 per NECT course) and logistics (the same 40-kg kit now contains materials for four NECT courses instead of two eflornithine treatments; Simarro *et al*., 2011; Yun *et al*., 2010). In terms of disadvantages, although the new NECT regimen is simpler than eflornithine alone, its remains complicated and resource-intensive (Yun *et al*., 2010). Furthermore, nifurtimox is not free from side effects, such

as gastrointestinal (nausea, vomiting, abdominal pain, anorexia) and neurological disturbances (convulsions, tremor and agitation, and occasionally – peripheral neuropathy), as well as allergic skin reactions. However, the adverse effects of nifurtimox are rarely life-threatening, and only some gastrointestinal disturbances were found to be significantly increased in NECT compared to eflornithine monotherapy (Burri and Brun, 2009; Fries and Fairlamb, 2003; Priotto *et al*., 2009).

1.2.3. Potential novel chemotherapeutics for the treatment of HAT

The chemotherapeutic agents that are currently available against sleeping sickness are at best ineffective and at worst – highly toxic. Therefore, if elimination of HAT as a public health risk is to be achieved, as set by the World Health Organisation (Simarro *et al*., 2011), new and improved drugs will be required, since the current strategy of re-analysing and optimising existing regimens and combinations thereof seems to have reached its peak with the implementation of NECT. In an effort to discover and develop novel therapeutics, the Drugs for Neglected Diseases *initiative* (DND*i*) has brought forward into clinical development another nitroaromatic compound, similar to nifurtimox – the 5-nitroimidazole fexinidazole (Hoechst 239). Fexinidazole entered phase I clinical trials in 2009 for use against second-stage sleeping sickness and is expected to progress to phase II clinical trials in the near future. Furthermore, compounds from two other chemical classes (oxaboroles and diamidines) are currently in pre-clinical development for potential use against sleeping sickness (Barrett, 2010).

1.3. Nitroaromatic pro-drugs

1.3.1. A historical overview

In recent years, interest in the use of nitroheterocyclic compounds as chemotherapeutic agents appears to be on the increase, not only for use against African sleeping sickness

but also against other infectious diseases, and even in cancer chemotherapy. For example, the 4-nitroimidazo-oxazine PA-824 is currently under clinical investigation for the treatment of tuberculosis (Ginsberg *et al*., 2009), whereas the 5-nitrothiazole nitazoxanide (Alinia®) has completed phase II clinical trials for the treatment of hepatitis C (Keeffe and Rossignol, 2009). Another drug, the dinitrobenzene CB1954, has also recently completed phase I and II clinical trials as part of a virus-directed enzyme pro-drug therapy (VDEPT) for prostate cancer (Patel *et al*., 2009). However, nitroaromatic drugs are not new to the medical scene. Interest in the chemotherapeutic properties of nitroaromatics was sparked by the discovery of Stillman and colleagues (Dodd *et al*., 1944) that addition of a nitro group (-NO_2) to the 5-position of simple furan derivatives significantly increases their antibacterial activity. The first nitroaromatic compound to be used for its antimicrobial properties was the 5-nitrofuran derivative nitrofurazone (5-nitro-2-furaldehyde semicarbazone; Furacin), which was successfully applied as a topical agent for the treatment of wounds during the second world war (Miura and Reckendorf, 1967). Shortly afterwards (1946 – 1951), nitrofurazone was found to have antitrypanosomal activity in experimental mouse models of infection (Grunberg and Titsworth, 1973; Williamson, 1962). These findings, combined with the knowledge that the drug was orally active and can enter the CNS, allowed nitrofurazone to proceed into clinical trials during the 1950s for the treatment of both *T. b. gambiense* and *T. b. rhodesiense* patients, most of whom were already refractory to pentamidine, suramin and organoarsenicals. Although nitrofurazone elicited cure in approximately half of these “hopeless” patients, who would otherwise have died for lack of alternative treatment, the high toxicity of nitrofurazone did not justify its further use in humans, other than in its original topical application (Miura and Reckendorf, 1967; Williamson, 1962; Williamson, 1970). During the 1960s, several other nitrofurans, including furaltadone (Altafur), were found to have antitrypanosomal

activity but were subsequently abandoned because of insufficient effectiveness and high neurotoxicity. Similar to nitrofurazone, these compounds were also found to cause haemolytic anaemia in patients with the glucose-6-phosphate dehydrogenase deficiency trait commonly found in Africa (Grunberg and Titsworth, 1973; Pépin and Milord, 1994; Williamson, 1970). However, hope for the use of nitrofurans against HAT was renewed in the early 1970s, when nifurtimox was shown to be better tolerated and more effective than nitrofurazone against *T. cruzi* infections in animals (Haberkorn and Gonnert, 1972) and to be active against acute Chagas' disease in humans (81% cure rate; Wegner and Rohwedde, 1972a). Following from these findings, the activity of nifurtimox against *T. b. gambiense* was demonstrated first in rats and then in a cautious clinical trial in four European *T. b. gambiense* patients (Janssens and Demuynck, 1977). Although subsequent clinical trials with various nifurtimox regimens during the 1980s (reviewed by Bouteille *et al*., 2003 and Pépin and Milord, 1994) did not report incidences of haemolytic anaemia, the efficacy of the drug against *T. b. gambiense* was variable (30 – 80% cure rate) and its toxicity increased with higher doses and prolonged treatment (Pépin *et al*., 1992). Consequently, nifurtimox never received approval as a monotherapy against HAT and was left only as a last resort drug for second-stage HAT patients refractory to all other available treatments. Finally, some two decades later, nifurtimox found its place in the chemotherapy of sleeping sickness as part of a combination therapy with eflornithine that was added to the Model List of Essential Medicines of the World Health Organisation in 2009 (Yun *et al*., 2010).

In parallel with nitrofurans, a similar class of aromatic compounds – the nitroimidazoles, was also investigated in terms of antimicrobial activity. The first indications that nitroimidazoles are chemotherapeutically active were given in the early 1950s, when Japanese scientists isolated the natural antibiotic azomycin and revealed its chemical structure as a 2-nitroimidazole. Although azomycin was too toxic for clinical

application, its efficacy against infections with the microaerophilic protozoan *Trichomonas* prompted further research. By the end of the 1950s, a whole series of nitroimidazoles had been synthesised, which included metronidazole (2-methyl-5-nitroimidazole-1-ethanol; Flagyl) among others (Grunberg and Titsworth, 1973; Raether and Hänel, 2003). This drug was one of the first nitroimidazoles to be successfully introduced into clinical practice, and remains widely applied for the treatment of microaerophilic protozoal and anaerobic bacterial infections (Löfmark *et al*., 2010). The effect of metronidazole on *T. b. rhodesiense* appears to have been investigated almost immediately after the drug was synthesised – as early as 1961[*]. However, to our knowledge, since this first report, there have only been a few studies on the use of metronidazole against sleeping sickness (caused by *T. b. rhodesiense*), which appeared in the period 1985 – 1996 and which indicated that this drug alone was not able to clear CNS infections even at toxic doses (see references in Foulkes, 1996). Similar to metronidazole, most nitroimidazole compounds tend to be most active against various anaerobic bacteria and protozoa, but are relatively inactive against aerobic organisms (Grunberg and Titsworth, 1973). Nevertheless, there are some exceptions to this, notably the 2-nitro derivative benznidazole (Roche 7-1051; Rochagan; Radanil), which was introduced in the 1970s for the treatment of South American trypanosomiasis. Benznidazole is still the drug of choice against Chagas' disease, as it is better tolerated than nifurtimox (Gutteridge, 1985; Rassi *et al*., 2010). However, it is ineffective against the African trypanosome, as demonstrated in a chronic mouse model of infection (Poltera *et al*., 1981). Another nitroimidazole that was synthesised in the 1970s, fexinidazole, was initially reported to have a very good activity against both *T. cruzi* and *T. brucei* infections in mice, in addition to its high efficacy against anaerobic

[*] The following article, which could not be accessed, was the oldest found in a PubMed search for "metronidazole" and one of the following terms "*Trypanosoma*", "African" or "sleeping sickness": Adriaenssens K. (1961) [Metronidazole in a case of sleeping sickness caused by *Trypanosoma rhodesiense*]. *Ann. Soc. Belg. Med. Trop. 41*, 393-395, in Dutch.

protozoa (Jennings and Urquhart, 1983; Raether and Seidenath, 1983). However, fexinidazole was abandoned in its pre-clinical development due to unspecified toxicity issues (Gutteridge, 1985). Similar "toxicity and commercial considerations" precluded the development of other 5-nitroimidazoles with antitrypanosomal activity that were investigated during the 1980s and 1990s, including MK(Merk) 436 and megazol (CL 64855) (Barrett, 2010; Gutteridge, 1985; Jennings, 1993). Despite the suspected mutagenicity of fexinidazole, the need for improved chemotherapy of sleeping sickness has led to the recent re-evaluation of this compound. The study by DND*i* revealed that fexinidazole is indeed safe in mammalian systems and confirmed the efficacy of the drug against all subspecies of *T. brucei* and against both stages of sleeping sickness. Consequently, after many years on the shelf, fexinidazole has finally moved from pre-clinical development into clinical trials for use against HAT (Torreele *et al*., 2010).

1.3.2. Mode of action and activation of nitroaromatic drugs

The available scientific evidence indicates that the nitro group is essential for the mode of action of nitroaromatic compounds, which are believed to act as pro-drugs and to require enzyme-mediated reduction of the nitro group to become cytotoxic. This reduction can proceed via one- or two-electron transfer (**Figure 1.3**). In the case of one-electron reactions, a nitro anion radical ($-NO_2^{\cdot -}$) is formed that is highly unstable and, under anaerobic conditions, can react with and cause damage to various intracellular components (Marr and Docampo, 1986). However, in the presence of molecular oxygen, the nitro radical is oxidised to the parental nitro pro-drug, generating a superoxide anion ($O_2^{\cdot -}$). This process is often referred to as futile redox cycling (Viode *et al*., 1999). Dismutation of the superoxide anion can lead to the formation of other reactive oxygen species, such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH), which can be highly damaging to the cellular components (Docampo *et al*.,

1981). If the capacity of the cells antioxidant defence system is overwhelmed, the build-up of reactive oxygen species can cause oxidative damage to proteins and lipids and eventually lead to cell death. For example, in *T. cruzi* the accumulation of high levels of hydrogen peroxide has been implicated in the mode of action of nifurtimox (Docampo and Moreno, 1986).

Similar to the African trypanosome, *T. cruzi* are thought be highly susceptible to oxidative stress, as they lack catalase and glutathione. Instead, removal of hydrogen peroxide and maintenance of the intracellular thiol redox balance depend exclusively on a unique for the kinetoplastids co-factor, termed trypanothione (bis-glutathionyl-spermidine) (Docampo, 1990; Fairlamb and Cerami, 1992). In some instances, futile redox cycling of nitroaromatic pro-drugs can be regarded as a form of detoxification, providing that the cell is able to cope with the resulting oxidative stress. For example, futile cycling of benznidazole in *T. cruzi* is a form of detoxification, as it prevents the built-up of the toxic nitro anion radicals and occurs sufficiently slowly to allow effective removal of the superoxide anion (Marr and Docampo, 1986). A similar mechanism of detoxification by redox cycling has also been proposed to operate in some strains of *Trichomonas*, which are resistant to metronidazole only under aerobic conditions (Townson *et al*., 1994).

There is also a third possibility for the nitro radical – under anaerobic conditions, two such radicals may react to form one molecule of the original pro-drug and one molecule of the toxic nitroso (-NO) derivative (Marr and Docampo, 1986). Alternatively, a nitroso derivative can be formed either via a sequential one-electron transfer or via direct two-electron reduction of the parent pro-drug. Usually, the nitroso intermediate is reduced in further two-electron transfer steps to form hydroxylamino (-NHOH), and in some cases amino ($-NH_2$) derivatives (Roldan *et al*., 2008). In theory, the nitroso and hydroxylamino compounds may also undergo one-electron reductions,

but there is no evidence for these reactions in biological systems (Docampo and Moreno, 1986). However, unstable hydroxylamino derivatives can disproportionate to form a toxic nitroso and an amino derivative (Tang *et al*., 2005). Finally, hydroxylamino derivatives may react non-enzymatically with thioesters, such as acetyl CoA, to form N-acetoxy species. The latter can act as alkylating agents and bind covalently to DNA or cause DNA interstrand crosslinking (Knox *et al*., 2003; Tang *et al*., 2005).

The intracellular fate of nitroaromatic pro-drugs is largely dependent on the availability of reducing enzymes, which vary with the organism and have different redox potentials. One-electron transfer reactions are typically carried out by oxygen-sensitive oxidoreductases, commonly referred to as type II nitroreductases. Typical type II nitroreductases include xanthine oxidase, glutathione reductase, cytochrome *c* reductase and cytochrome P450 reductase in aerobic organisms, as well as hydrogenase and ferredoxin:pyruvate oxidoreductase in anaerobic protozoa (Koder *et al*., 2002; Townson *et al*., 1994). Conversely, two-electron transfers are carried out by type I, oxygen-insensitive nitroreductases. With the exception of some few mammalian enzymes, such as DT-diaphorase and xanthine dehydrogenase, type I nitroreductases are usually bacterial flavoproteins, which use NADH or NADPH as a co-factor (Koder *et al*., 2002; Roldan *et al*., 2008).

Due to the exclusive use of nifurtimox and benznidazole in the treatment of South American trypanosomiasis, trypanosomal enzymes involved in the activation of nitrodrugs have been studied primarily in *T. cruzi*. A number of enzymes involved in redox reactions have been proposed to metabolise nitro pro-drugs in *T. cruzi*, including old yellow enzyme (*Tc*OYE, prostaglandin $F_{2\alpha}$ synthase; Kubata *et al*., 2002), trypanothione reductase (*Tc*TryR), lipoamide dehydrogenase and several NADPH:cytochrome P450 reductases (Blumenstiel *et al*., 1999; Henderson *et al*., 1988;

Portal *et al*., 2008; Viode *et al*., 1999). It has been proposed that *Tc*OYE is the most important enzyme in activation of nitro pro-drugs, as evidenced by its loss in *T. cruzi* with *in vitro*-induced benznidazole resistance (Murta *et al*., 2006), and by the inhibition of all detectable nitroreductase activity in *T. cruzi* lysates treated with anti-*Tc*OYE antibodies (Kubata *et al*., 2002). However, a homologue of *Tc*OYE is lacking in *T. brucei*, which has been confirmed by the sequencing of the *T. brucei* genome (Berriman *et al*., 2005). Unexpectedly, the genome sequencing project of *T. brucei* also revealed the presence of a putative nitroreductase gene (Tb927.7.7230; *Tb*NTR), similar to type I bacterial nitroreductases. The role of this nitroreductase in nitro pro-drug activation formed the basis of this current study. During the course of this study, Wilkinson and co-workers reported that laboratory-induced resistance to nifurtimox was associated with loss of the *Tb*NTR homologue in *T. cruzi*, confirming a role of *Tc*NTR in nitro pro-drug activation (Wilkinson *et al*., 2008). This, and subsequent work by Wilkinson and colleagues will be discussed in more detail in other chapters.

1.3.3. Resistance to nitroaromatic drugs in trypanosomes

Natural variations in the susceptibility of *T. cruzi* to nitroaromatic compounds were already evident when nifurtimox was first introduced for the treatment of Chagas' disease in the 1960s, although the occurrence of natural resistance to nitroaromatics in *T. cruzi* was disputed at the time (Haberkorn and Gonnert, 1972; Wegner and Rohwedde, 1972b). However, subsequent characterisation of domestic and sylvatic isolates of *T. cruzi* revealed that some strains, which have not been exposed to drug pressure, were naturally resistant to both nifurtimox and benznidazole (Filardi and Brener, 1987).

There is evidence that natural variation in susceptibility to nitroaromatics may also occur in *T. brucei*. For example, when nitrofurazone was initially tested in animal

models of HAT (in guinea pigs and mice), it was noticed that after a single dose, its efficacy was strain- and subspecies-dependent, and that *T. b. rhodesiense* were generally less susceptible than *T. b. gambiense*. Furthermore, mice infected with a certain isolate of *T. b. rhodesiense* could not be cured by nitrofurazone (Miura and Reckendorf, 1967; Williamson, 1962). Variability in the efficacy of nitrofurazone has also been observed in humans, with cure elicited in approximately half of the treated patients (Williamson, 1962). Likewise, nifurtimox was found to vary hugely (30 – 80%) in its efficacy in clinical trials with *T. b. gambiense* patients. Although these trials used different doses and regimens of the drug, the range of variation led to speculation that there may also be regional differences in the nifurtimox susceptibility of *T. b. gambiense* (patients in Zaire were more refractory than patients in Sudan; Pépin *et al*., 1992). This was supported by recent studies with clinical isolates of *T. b. gambiense*, which revealed that there is a 10-fold variation in their *in vitro* susceptibility to nifurtimox (0.34 – 3.8 μM; Likeufack *et al*., 2006; Maina *et al*., 2007).

Undoubtedly, nitro drug resistance is understudied in *T. brucei*. However, with the recent introduction of NECT as part of the chemotherapeutic arsenal against sleeping sickness and with ongoing clinical trials of fexinidazole for use against both stages of HAT, there is now a pressing need to understand the potential of these organisms to acquire nitro drug resistance in the field.

1.4. Project aims

The overall aim of this project is to gain a better understanding of the potential for nitro drug resistance in Africa trypanosomes. The specific aims are:

- to examine the *in vitro* and *in vivo* resistance potentials of nifurtimox and fexinidazole in BSF *T. brucei*, and
- to investigate the involvement of *Tb*NTR in drug activation of nitroheterocycles.

Chapter 2 -

Materials and methods

2.1. Materials

Initial studies with fexinidazole were carried out with compound kindly provided by Professor Frank W. Jennings, University of Glasgow. Fexinidazole used in subsequent studies, together with fexinidazole sulfoxide, fexinidazole sulfone and PA-824 were synthesised in our laboratory by Dr Stephen Patterson as previously described (Sokolova *et al*., 2010). The following compounds were kindly donated to Professor Alan H. Fairlamb:

- nifurtimox from Bayer, Argentina
- benznidazole from Dr Ralph A. Neal, London School of Hygiene & Tropical Medicine
- metronidazole from The Boots Company PLC, Nottingham
- eflornithine from Marion Merrell Dow Pharmaceuticals Inc., Cincinnati, Ohio

All other chemicals were of the highest grade and purity available commercially. Stocks of nitroaromatic compounds were prepared in DMSO (supplied by BDH). Buffers and other reagents were prepared with ultrapure (Type 1, 18.2 MΩ cm) Milli-Q® water, unless otherwise stated.

2.1.1. Analysis of nitroaromatic compounds

The identity of all compounds synthesised in-house was confirmed by ^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR (PA-824 only) and high resolution MS, performed by Dr Stephen Patterson. All compounds were determined to be >95% pure by liquid chromatography (LC)-MS and the optical purity of PA-824 was confirmed by optical rotation measurements (Sokolova *et al*., 2010). Solubility screenings by nephelometry, which measures the concentration of particulate matter as a function of the intensity of scattered light (Agarwal and Berglund, 2004), were carried out in collaboration with Suzanne Norval, Drug Metabolism and Pharmacokinetics (DMPK) group, University of Dundee. The solubility of PA-824, fexinidazole, fexinidazole sulfoxide and fexinidazole

sulfone in 0.5% (v/v) DMSO in HMI-9T culture media (section 2.5.1) was determined in a 96-well format, using a published high-throughput method (Bevan and Lloyd, 2000). Measurements were performed using BMG Labtech NEPHELOstar Galaxy laser nephelometer, which measures the forward-scattered light when a laser beam is directed through a solution. Data were analysed using the MARS analysis software (version 1.11, BMG Labtech).

2.2. Molecular biology

2.2.1. Isolation of genomic DNA from *T. brucei*

Genomic DNA (gDNA) was isolated from 50 – 100 ml late-log phase BSF *T. brucei* S427 ($1 \times 10^6 – 3 \times 10^6$ cells ml^{-1}). Cells were harvested by centrifugation (800 x *g*, 10 min, room temperature (RT)) and lysed overnight at 56°C in 450 µl TENS buffer (10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 100 mM NaCl, 0.5% (w/v) SDS) containing 0.1 mg ml^{-1} proteinase K. DNA was extracted with Tris-buffered (pH 8.0) phenol/chloroform/isoamyl alcohol (PCI, 25:24:1). The organic and aqueous phases were separated by centrifugation at 16,000 x *g* for 1 min, RT. The upper (aqueous) phase containing gDNA was subject to a second extraction with PCI and a final extraction with CI (24:1). To separate gDNA from inorganic contaminants, PCI-extracted DNA was precipitated and washed twice in 70% (v/v) ethanol. Finally, DNA was allowed to air-dry to remove traces of ethanol. The purified gDNA was resuspended in 10 mM Tris-HCl, pH 8.5 and stored at 4°C.

2.2.2. DNA amplification

DNA was amplified by PCR using *Pfu* DNA polymerase and buffers (Promega) according to the manufacturer's guidelines. A typical 50-µl reaction contained 120 – 160 pmol of gene-specific forward and reverse primers (**Table 2.1**) and approximately

50 – 200 ng of template DNA. All PCRs were carried out in 0.2 ml thin-walled tubes (Thermo Scientific) in a Thermo-Hybaid MBS 0.2G thermal cycler with a heated lid. Usually, DNA was denatured at 94°C for 10 min before the addition of 3 – 6 U *Pfu* DNA polymerase. PCR was continued for 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 55 – 68°C depending on T_m of the primers) and extension (2 min per kb at 72°C). A final extension for 10 min at 72°C was included after the last cycle. PCR products were stored at 4°C.

2.2.3. Agarose gel electrophoresis and extraction of DNA

DNA size and purity were analysed by agarose gel electrophoresis. Gels were prepared by dissolving 0.8% (w/v) agarose in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) containing 0.1 μg ml^{-1} ethidium bromide for DNA staining. Separation was performed in TAE buffer at 80 V. DNA was visualised using either UVP AutoChemi transilluminator or U:Genius Gel Documentation System (Syngene). One Kb Plus DNA Ladder (Invitrogen) was used to determine the apparent size of the resolved DNA. When extraction of DNA from the agarose gel was required, the DNA band of interest was excised from the gel and purified with the QIAquick® Gel Extraction Kit (Qiagen) as per manufacturer's guidelines.

2.2.4. Cloning of PCR products

Following PCR (section 2.2.2) and, when necessary, gel extraction (section 2.2.3), blunt-end PCR products were usually sub-cloned into the *Vaccinia* virus DNA topoisomerase I (TOPO®)-activated pCR®-BluntII-TOPO® vector (Invitrogen). Alternatively, blunt-end PCR products were cloned in the directional expression vector pET151/D-TOPO® (Invitrogen), by means of a 3′-overhang present on the non-coding vector strand. Ligations were carried out for 30 min on ice, following the

manufacturer's guidelines. Ligated TOPO® vectors were immediately transformed into 50 µl TOP10 chemically competent cells (**Table 2.2**), as described in section 2.2.5.

2.2.5. Transformation of competent *E. coli* cells

Chemically competent *E. coli* cells (see **Table 2.2** for an exhaustive list) were transformed by heat shock. Briefly, cells (10 – 50 µl aliquots) were allowed to thaw on ice for 10 min, before the addition of 50 ng plasmid DNA. Alternatively, 5 – 10 µl of a typical ligation reaction (sections 2.2.4 and 2.2.9) was added to the cells. Following 30 min incubation on ice, cells were heat-shocked for 45 s at 42°C and immediately transferred on ice for 2 min. The only exception was ArcticExpress™(DE3)RP competent cells, which were incubated with DNA in the presence of 2-mercaptoethanol and were heat-shocked for 20 s. Transformed cells were incubated with 250 µl of the nutrient rich SOC media* for 20 – 75 min at 37°C. Finally, 50 – 200 µl aliquots of the transformed cells were plated on Luria-Bertani (LB) agar plates containing the appropriate selection antibiotics, and incubated at 37°C overnight.

2.2.6. Purification of plasmid DNA

Plasmid DNA was isolated from bacterial cells using the QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's guidelines. Briefly, TOP10 or JM109 *E. coli* cells (**Table 2.2**) were transformed with the plasmid of interest as described in section 2.2.5. Single colonies were incubated overnight at 37°C in 5 ml LB media with the appropriate selection antibiotics. Overnight cultures were harvest by centrifugation at 3800 x *g* for 10 min at RT. The bacterial pellets were lysed under alkaline conditions (based on the modified method of Birnboim and Doly, 1979). Following neutralisation,

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* All bacterial growth media and plates were prepared by Media Services at the University of Dundee, using standard protocols (Sambrook and Russell, 2001).

plasmid DNA was adsorbed on silica membranes in the presence of high salt (Vogelstein and Gillespie, 1979). Finally, the purified DNA was eluted in a low-salt buffer (10 mM Tris-HCl, pH 8.5) suitable for subsequent applications. Alternatively, for large scale preparations (up to 50 µg DNA), plasmid DNA was isolated in a similar way from 80 – 150 ml overnight culture using the HiSpeed® Plasmid Midi Kit (Qiagen).

2.2.7. DNA sequencing

DNA sequencing was performed by DNA Sequencing and Services™ at the University of Dundee (http://www.dnaseq.co.uk), using Applied Biosystems Big-Dye® Terminator chemistry version 3.1 on an Applied Biosystems 3730 automated capillary DNA sequencer. Plasmid DNA samples were submitted at concentrations of 16 – 20 ng μl^{-1}, whereas gel-purified PCR products were submitted at 40 ng μl^{-1}. All primers used in DNA sequencing are listed in **Table 2.1**.

2.2.8. Digestion of plasmid DNA with restriction endonucleases

Analysis of DNA with restriction enzymes was typically performed on 2 – 4 µl purified plasmid DNA (see above) in a 5 µl reaction. The total amount of restriction endonucleases (5 – 6 U, Promega or Fermentas FastDigest® enzymes) was maintained at 10% of the final volume for both single and double digests. Reactions were incubated at 37°C for 3 – 16 h and the resulting band pattern was visualised by gel electrophoresis (section 2.2.3). For preparative digests, up to 2 µg of plasmid DNA was digested with a total of 20 – 25 U of restriction enzymes in a final volume of 25 – 60 µl. Reactions were incubated for at least 5 h at 37°C. Preparative digests of cloning vectors were incubated for an additional 1 h in the presence of Shrimp alkaline phosphatase (SAP, 1 U, Promega). Immediately following the incubation, SAP was heat-inactivated for 15 min

at 65°C. Prior to ligation, either the vector or insert of interest was purified by gel-extraction as previously described (section 2.2.3).

2.2.9. Ligation

Ligation reactions were carried out with gel-purified restriction fragments (section 2.2.3 and 2.2.8). Purified target vector (~50 ng) and insert were typically combined in molar ratios of 1:1, 1:3 and 1:5 respectively, in a 10-µl reaction. To control for background vector self-ligation, 50 ng of vector was incubated in the absence of insert. Ligations were carried out in the presence of 1 U T4 DNA ligase (Promega or Roche) at RT for 5 h or at 14°C overnight. Following incubation, 5 – 10 µl of ligation reaction was transformed in 30 – 50 µl XL10-Gold® Ultracompetent cells as in section 2.2.5. Single colonies of the transformed cells were used for plasmid purification (section 2.2.6), and the accuracy of the assembled constructs was verified by restriction digest and DNA sequencing as previously described. A list of the resulting ligation products used for bacterial expression studies is given in **Table 2.3**.

2.2.10. Replacement of the thrombin recognition site in pET43.1c_*Tb*NTRΔ83

The sequence encoding the thrombin protease recognition motif was replaced with the Tobacco Etch Virus (TEV) protease recognition motif using oligonucleotides TEV_forward and TEV_reverse (**Figure 2.1**). The two oligonucleotides (4 nmoles each) were heated to 99°C and annealed by slow cooling to RT over 5 – 6 h. The annealed DNA was phosphorylated at the 5′-end using T4 polynucleotide kinase (Promega) as per manufacturer's guidelines and ligated into the SacI/SacII-digested vector as described in section 2.2.9. Replacement of the recognition motif was confirmed by restriction digest and DNA sequencing.

2.2.11. Southern analysis of genomic DNA

Genomic DNA isolated from wild-type and nitrodrug-resistant *T. brucei* was analysed by Southern blot (Southern, 1975). DNA (1 – 5 μg) was digested overnight at 37°C in the presence of various restriction enzymes (Promega). The digested DNA was resolved on a 0.8% (w/v) agarose gel (section 2.2.3) and depurinated in 0.25 M HCl for 10 min. Following equilibration in 0.4 M NaOH, DNA was transferred to a positively charged nylon membrane (GE Healthcare or Roche) by the downward capillary transfer method (Sambrook and Russell, 2001). When the transfer was complete, the membrane was rinsed in 5-fold SSC buffer (supplied as 20-fold buffer by Invitrogen) for 25 min and DNA was cross-linked to the membrane by UV irradiation at 1200 μJ cm^{-2}. If required, the cross-linked DNA was visualised using Methylene Blue (Amresco®) DNA stain (1-fold in Milli-Q® water, filter sterilised), followed by washing with 0.1% (w/v) SDS. The membrane was pre-hybridised at 42°C for 2 h in DIG Easy Hyb (Roche) prior to overnight hybridisation at 42°C with the denatured digoxigenin-dUTP-labelled DNA probe.

Probes were prepared in advance from the open reading frame (ORF) of *TbNTR*, using either DIG-High Prime DNA Labelling and Detection Starter Kit II (Roche) or, in later experiments, PCR DIG Probe Synthesis Kit (Roche), following the manufacturer's guidelines. The labelled ORFs of dihydrofolate reductase-thymidylate synthase (*TbDHFR-TS*) and trypanothione synthetase (*TbTryS*) from *T. brucei* S427 (section 2.5.1) were courtesy of Dr Susan Wyllie, Dr Sandra L. Oza and Dr Natasha Sienkiewicz (Sienkiewicz *et al*., 2008; Wyllie *et al*., 2009).

Washing of the membrane post hybridisation, as well as the immunological detection of the probe, were performed with the DIG Wash and Block Buffer Set and the CSPD ready-to-use reagent (Roche), according to the manufacturer's guidelines. The processed membrane was exposed to Amersham Hyperfilm ECL™ (GE Healthcare)

for 2 min. To re-use the membrane for subsequent hybridisations, the membrane was rinsed in autoclaved ultrapure Milli-Q® water and stripped with 3 x 50 ml 0.4 M NaOH, 1% (w/v) SDS at 45°C over a total of 50 min. The stripped membrane was neutralised in 2 x SSC buffer and either stored at 4°C or pre-hybridised and processed further, as described.

2.3. Expression and purification of recombinant proteins

2.3.1. Expression of recombinant *Tb*NTR

Expression constructs (**Table 2.3**), carrying full-length or truncated versions of *TbNTR*, were transformed into a number of expression strains of *E. coli* (**Table 2.2**) as described in section 2.2.5. Cells transformed with empty vectors were processed in parallel and used as expression controls. Single colonies were selected and incubated overnight at 37°C in 10 – 100 ml LB media supplemented with the appropriate selection antibiotic. Overnight cultures were harvested by centrifugation (3800 x *g*, 10 – 15 min, RT), resuspended in one volume of fresh media with antibiotics, and diluted 20- to 100-fold in either autoinduction or LB media with selection antibiotics. Cells in autoinduction media were incubated with agitation at 200 rpm for 1 h at 37°C, followed by 24 – 48 h incubation at the selected expression temperature. Cells in LB media were incubated at 37°C until the optical density at λ = 600 nm (OD_{600}) reached 0.5 – 0.8. For expression at 37°C, cells were induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, 0.2 μm filter sterilised) and incubated further, up to 20 h. Alternatively, cells were equilibrated for 30 – 60 min at the selected expression temperature ($OD_{600} \approx$ 1.2 – 1.8) prior to addition of 1 mM IPTG. Following incubation, cells were harvested by centrifugation (4,000 x *g*, 30 – 45 min, 4°C). Cell pellets were washed once with PBS and either processed as described in section 2.3.2, or flash frozen in dry ice/ethanol and stored at -80°C.

2.3.2. Preparation of cell-free bacterial lysates

All procedures were carried out on ice unless otherwise specified. Following expression of recombinant protein, cell pellets were resuspended in the appropriate lysis buffer (**Table 2.4**) supplemented with DNAse I (Sigma) and a complete EDTA-free protease inhibitor cocktail (Roche). Typically, cells were lysed using a One-Shot or a Continuous Cell Disruptor (Constant Systems) under pressure of 30 kpsi. Alternatively, samples of ≤2 ml were lysed in the presence of lysozyme (from chicken egg white, Sigma) by 5 cycles of flash-freezing and rapid thawing, followed by sonication (3 x 15 – 30 s, with intermittent cooling) using a Soniprep 150 sonicator (Sanyo) at 15 μm amplitude with an exponential probe. When required, bacterial cell lysates were clarified by centrifugation (10,000 – 50,000 x *g*, 10 – 45 min, 4°C).

2.3.3. Immobilised metal affinity chromatography (IMAC)

Nickel affinity chromatography was performed using a 5 ml HisTrap™ HP* pre-packed column, pre-equilibrated in 10 column volumes of binding buffer (lysis buffer supplemented with ≤40 mM imidazole, **Table 2.4**). Clarified bacterial cell lysates (section 2.3.2) were applied to equilibrated columns at a constant flow rate (2 – 2.5 ml min^{-1}) using a peristaltic pump and the flow-throughs were collected. Columns were connected to an ÄKTApurifier™ 100 FPLC system (GE Healthcare), and washed in 10 – 20 column volumes binding buffer. Bound proteins were eluted at a flow rate of 5 ml min^{-1} with a 0 – 100% (v/v) gradient of 1 M imidazole in binding buffer. Elution was monitored at λ = 280 nm and 2-ml fractions were collected using the in-built Frac-950 automated fraction collector. Samples of loaded, flow-through and eluted proteins were analysed by SDS-PAGE (section 2.4.2), and when necessary, by Western blotting (section 2.4.3).

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* All HisTrap™, HiTrap™ and HiLoad™ columns were purchased from Amersham/GE Healthcare.

2.3.4. Ion exchange chromatography

Protein separation by anion exchange chromatography was performed on a small scale using 5 ml HiTrap™ Q HP and 5 ml HiTrap™ Q FF columns. Large scale anion exchange was carried out in a similar manner using a HiLoad™ 16/10 Q Sepharose® HP column. Cation exchange chromatography was carried out only on a small scale, using a 1 ml HiTrap™ SP FF column. Columns were pre-equilibrated in a low-salt binding buffer (ionic strength ≤100 mM), prior to sample loading. Small-scale samples were loaded manually with a syringe. Following column washing (~10 column volumes binding buffer), proteins were eluted with a three-step gradient of 0 – 1 M NaCl in binding buffer. Flow-through and elution steps were collected for analysis. Alternatively, large samples were loaded on the column by automated sample injection using an ÄKTApurifier™ 100 FPLC. Column washing, protein elution and fraction collection were performed as in section 2.3.3, with a 0 – 100% (v/v) continuous gradient of 1M NaCl in binding buffer at 2 ml min^{-1}. All samples were analysed by SDS-PAGE/Western blotting as described.

2.3.5. Size exclusion chromatography

Protein separation by gel filtration was performed using either HiLoad™ 26/60 Superdex™ 75 or HiLoad™ 16/60 Superdex™ 200 prep grade columns. Column equilibration, automated sample loading and protein separation were carried out according to the manufacturer's guidelines. Fractions (2 ml) were collected and analysed as previously described (section 2.3.3). Following each run, gel filtration standards (Bio-Rad) were applied to the column and resolved under the same conditions as the sample. To determine the molecular weight (MW) of the resolved samples, a calibration curve was prepared by plotting log MW for each gel filtration standard as a function of its elution volume divided by the void volume of the column ($V_R \div V_0$).

2.3.6. Other chromatographic methods

In addition to the standard IMAC (section 2.3.3) and ion exchange chromatography (section 2.3.4), several other methods were tested in attempts to purify recombinant *Tb*NTR$_{\Delta 54}$-His$_6$. Initially, a protein preparation in 20 mM sodium phosphate, pH 7.0, was applied to a 1 ml Co-MAC™ column (Novagen), which is an IMAC column pre-charged with Co^{2+} ions in place of Ni^{2+}. The column was washed manually in 6 column volumes of binding buffer and bound proteins were eluted in 250 mM imidazole in binding buffer.

Alternatively, an identical preparation was applied to a 5 ml HiTrap™ Blue HP column, which not only binds proteins via hydrophobic and electrostatic interactions, but also mimics nucleotide co-factors (e.g. NAD^+) and provides an alternative method for affinity chromatography. Following a washing step (10 column volumes binding buffer), proteins bound to HiTrap™ Blue HP were eluted manually in 2 M NaCl. Aliquots from all steps were taken and analysed as described (section 2.4.2 and 2.4.3).

2.3.7. Batch affinity chromatography

Clarified bacterial cell lysates (10,000 x *g*, 30 min, 4°C; section 2.3.2) of *E. coli* BL21-CodonPlus®(DE3)-RP expressing *Tb*NTR$_{\Delta 54}$-His$_6$ (section 2.3.1) were incubated with 10% (v/v) Ni-NTA (nitrilotriacetic acid) agarose beads (Qiagen) for 2 h at 4°C with gentle agitation. Cells transformed with an empty pET15b_TEV vector were used to control for non-specific protein interactions with the beads. Binding was carried out in the presence of 50 mM sodium phosphate, pH 7.8 or in NaCl/sucrose/CHAPS-buffer (50 mM sodium phosphate, pH 7.8 supplemented with 0.5 M NaCl, 40% (w/v) sucrose and 1% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)). Unbound proteins were removed by centrifugation (1,000 x *g*, 10 s, RT) in 0.8 ml centrifuge columns (Thermo Scientific). Beads were washed with 20 column

volumes of 20 mM imidazole in phosphate buffer with 0.5 M NaCl, and proteins were eluted in 3 x 2 column volumes of 0.5 M imidazole in washing buffer with 0.5% (v/v) Triton® X-100. Samples were analysed by SDS-PAGE.

2.3.8. Buffer exchange and sample concentration

Buffer exchange was carried out by three different methods: dialysis, gel filtration and following sample concentration. The preferred method for dialysis was using Slide-A-Lyzer dialysis cassettes (Thermo Scientific) with a molecular weight cut-off (MWCO) of 3.5 kDa or 10 kDa and a capacity of 0.5 – 12 ml. Dialysis buffer (150- to 500-fold sample volume) was exchanged three times over 1.5 – 16 h. Samples of <0.5 ml were dialysed using D-Tube™ Mini Dialyzer (6 – 8 kDa MWCO; Novagen), according to the manufacturer's guidelines. Alternatively, desalting and/or buffer exchange of very small samples was performed by the principle of gel filtration using 0.5 ml Zebra™ Spin desalting columns (7 kDa MWCO; Thermo Scientific) according to the manufacturer's guidelines. Large (>10 ml) or dilute samples were concentrated by centrifugation at 3,800 x *g*, 4°C, using Vivaspin 20 concentrators (Sartorius Stedim Biotech) with 10 kDa MWCO and polyethersulfone membranes. For simultaneous buffer exchange, the concentrated sample was diluted in the required buffer and concentrated again. The procedure was repeated at least 3 times.

2.3.9. Proteolytic cleavage of affinity tags

Three types of proteases were used for the removal of fusion tags from recombinant proteins: thrombin, enterokinase and TEV protease. Thrombin (from bovine plasma; GE Healthcare) was incubated overnight at RT or 4°C with affinity purified (section 2.3.3) NusA-*Tb*NTR$_{\Delta 83}$ (1 – 10 U protease per 100 µg substrate (total protein)). Samples were removed at various times for analysis by SDS-PAGE. Alternatively, NusA-*Tb*NTR$_{\Delta 83}$

was digested at RT or 4°C with recombinant enterokinase (rEK, Novagen; 1 – 5 U per 100 μg), following the manufacturer's guidelines. Other fusion proteins (**Table 2.3**) were cleaved using His-tagged recombinant TEV protease produced in our laboratory, at a mass ratio between 1:5 and 1:25, protease to substrate respectively. Incubation at (23 – 37)°C was carried out for a maximum of 2 h, whereas incubation at (4 – 14)°C was carried out for 24 h.

2.3.10. Protein purification under denaturing conditions

Purification of recombinant His_6-*Tb*$NTR_{\Delta 7}$ from inclusion bodies was performed under denaturing conditions, using Pierce B-PER™ (Bacterial protein extraction reagent) and Inclusion Body Solubilisation Reagent (IBSR; Thermo Scientific). Recombinant protein was purified from BL21-CodonPlus®(DE3)-RP cells, following IPTG-induced expression (16°C, 62 h; section 2.3.1). Cell pellets were lysed over 20 min at RT in 5 ml B-PER per 1 g pellet, supplemented with DNAse I (Sigma) and a complete EDTA-free protease inhibitor cocktail (Roche). Soluble *E. coli* proteins were removed by centrifugation (27,000 x *g*, 15 min, 4°C) and inclusion bodies-containing pellets were washed once in B-PER. To remove further *E. coli* contaminants, pellets were sequentially washed in 2, 4 and 6 M urea in 100 mM sodium phosphate, pH 8.0, 10 mM Tris-HCl (5 ml per g pellet; method based on Reischl *et al*., 1996). Washed pellets were solubilised in IBSR (8 ml per g pellet) at 4°C overnight. Recombinant protein was then purified under denaturing conditions (5 M guanidinium-HCl in 50 mM Tris-HCl, pH 6.8, 0.3 M NaCl, 10% glycerol) by Ni^{2+}-affinity chromatography as described (section 2.3.3). Fractions were analysed by SDS-PAGE and fractions of the highest purity were pooled for use in subsequent experiments.

2.3.11. Expression and purification of recombinant *Ec*NfsB

The gene encoding *E.coli* NfsB was amplified by PCR from *E. coli* K12 gDNA, kindly provided by Professor William N. Hunter, University of Dundee. *Ec*NfsB, cloned into the pET-3a expression vector (section 2.2.9), was expressed in BL21-Gold(DE3) cells (**Table 2.2**) overnight at 20°C in autoinduction media, as previously described (section 2.3.1). Recombinant *Ec*NfsB was purified according to a published method (adapted from Anlezark *et al*., 1992). Briefly, clarified bacterial cell lysates (50,000 x *g*, 40 min, 4°C; section 2.3.2) were applied to a 5 ml HiTrap™ Phenyl HP column in the presence of 20 mM potassium phosphate, pH 7.0, 0.3 M $(NH_4)_2SO_4$. Flow-through fractions were collected, concentrated and dialysed against 20 mM Tris-HCl, pH 7.6 (section 2.3.8). Dialysed fractions were applied to a 5 ml HiTrap™ Q FF column and eluted with a 0 – 0.2 M KCl gradient over 10 column volumes, using ÄKTApurifier™ 100 FPLC as described (section 2.3.4). Fractions of the highest purity (section 2.4.2) and activity (section 2.4.5) were pooled and concentrated to 0.5 mg ml^{-1}. Aliquots were flash-frozen in the presence of 10 μM FMN and 20 % (v/v) glycerol, and stored at -80°C.

2.3.12. Cell-free expression of recombinant *Tb*NTR

Recombinant *Tb*$NTR_{\Delta 54}$-His_6 was expressed using the TNT® (transcription and translation) T7 coupled wheat germ extract system (Promega). To prepare DNA for use with this expression system, purified pET20b_TEV_*Tb*NTRΔ54 (section 2.2.6) was linearised by restriction digest with PvuI (Promega, section 2.2.7) and extracted with PCI (section 2.2.1). Following precipitation in 70% (v/v) ethanol, the linearised DNA was resuspended in nuclease-free water (Promega) to 0.5 μg μl^{-1}. An empty pET15b_TEV vector was used as a negative control and pET3a_*Ec*NfsB was used as a positive expression control. Expression was performed in the presence of RNasin® ribonuclease inhibitor (Promega), for 1.5 h at 30°C, according to the manufacturer's

guidelines for non-radioactive reactions. Immediately following expression, the reaction mix was incubated for 2 h at 4°C with 5% (v/v) Ni-NTA agarose resin (Qiagen). Unbound proteins were separated by centrifugation at 1,000 x *g*, 10 s, RT. Bound proteins were eluted in 0.5 M imidazole in 50 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 40% (w/v) sucrose. Protein expression and binding to the resin were analysed by Western blotting (section 2.4.3) and nitroreductase activity assays (section 2.4.5).

2.4. Protein detection and characterisation

2.4.1. Protein quantification

Proteins were quantified using the Bio-Rad protein assay (Bio-Rad) based on the method of Bradford (Bradford, 1976). The assay was performed according to the manufacturer's guidelines for the microassay procedure, using BSA as a standard. Absorbance at λ = 595 nm was measured in a UV-1601 Shimadzu spectrophotometer. Standard curves were prepared using UV Probe software (Shimadzu).

2.4.2. SDS-PAGE

SDS-PAGE (Laemmli, 1970) was used to determine the size and purity of recombinant proteins. Briefly, samples were boiled for 10 min in the presence of Laemmli sample buffer (Bio-Rad) containing either 5% (v/v) 2-mercaptoethanol or 10 mM dithiotreitol (DTT). Protein bands were resolved on a NuPAGE® Novex® 12% Bis-Tris gel (1.0 mm, Invitrogen), using the XCell *SureLock*™ Mini-cell electrophoresis apparatus (Invitrogen). Electrophoresis was carried out at 180 V in 1-fold NuPAGE® MES SDS running buffer (Invitrogen), until the desired separation was achieved. Gels were stained with Coomassie® Brilliant Blue [0.1% (w/v) G-250 or 0.01% (w/v) R-250, in 40% (v/v) methanol, 10% (v/v) acetic acid] and destained with 20% (v/v) methanol, 10% (v/v)

acetic acid. Mark12™ (Invitrogen), Broad Range (Bio-Rad) and Precision Plus™ (All Blue, Bio-Rad) protein standards were used to determine the MW of the resolved bands.

2.4.3. Western blotting

Prior to analysis by Western blotting, unstained SDS-PAGE gels (section 2.4.2) were equilibrated in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol; Towbin *et al*., 1979). Proteins were transferred onto Protran™ nitrocellulose membrane (Whatman) using Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad) at 25 V for 20 min. To block non-specific binding sites, membranes were incubated for at least 1 h with 5% (w/v) dry milk in PBS containing 0.5% (v/v) Tween® 20. Primary and secondary (horseradish peroxidise (HRP)-conjugated) antibodies (**Table 2.5**) were incubated with the blot for 1 h each in PBS containing 1% (w/v) dry milk and 0.5% (v/v) Tween® 20, with an intermediate washing step (3 x 10 min) in antibody dilution buffer. Finally, membranes were washed (3 x 10 min) in buffer without milk. Alternatively, immunological detection was performed using the His•Tag® Antibody HRP Conjugate Kit (Novagen) according to the manufacturer's guidelines, with alkali-soluble casein as a blocking reagent and PBS with or without 0.5% (v/v) Tween® 20 as a washing buffer. Washed blots were incubated with Amersham ECL™ or ECL Plus™ detection reagent (GE Healthcare) and exposed to Hyperfilm ECL™ (GE Healthcare), following the manufacturer's protocol.

2.4.4. Protein fingerprinting analysis

Identification of proteins by mass fingerprinting analysis was performed by the FingerPrints Proteomics Facility at the University of Dundee. Samples were submitted to the facility resolved on a NuPAGE® Novex® 12% Bis-Tris gel (section 2.4.2) and stained with SimplyBlue™ Safe Stain (Invitrogen). Protein bands of interest were

excised from the gel and trypsin-digested, prior to analysis by nano-LC coupled to electrospray ionisation tandem MS (ESI-MS-MS), using the 4000 QTRAP (Applied Biosystems) tandem MS system.

2.4.5. Nitroreductase activity in bacterial lysates

The nitroreductase activity of bacterial cell lysates or protein preparations was measured according to a modified nitroreductase activity assay (based on Zenno *et al*., 1996). Briefly, assays were performed in 50 mM sodium phosphate, pH 7.0, 1% (v/v) DMSO, at 27°C in 1 ml acrylic cuvettes (1-cm path length, BrandTech Scientific). Protein preparations (up to 300 µl) were equilibrated to 27°C for 1 min in the presence of 100 µM nitrofurazone (Sigma) and background reduction of nitrofurazone was recorded for further 2 min, using a Shimadzu UV-1601 spectrophotometer equipped with a temperature-regulating Peltier device. The reaction was initiated with 100 – 250 µM NADH or NADPH (Melford Laboratories) and the initial rate of nitrofurazone disappearance was monitored for up to 1 min at λ = 420 nm ($\varepsilon_{420\ nm}$ = 5,590 M^{-1} cm^{-1}; Race *et al*., 2005). One unit of nitroreductase activity was defined as the amount of protein required to reduce 1 µmol of nitrofurazone per minute. The change in moles of substrate (n) was derived from the initial change in absorbance over time ($\Delta A/\Delta t$), based on Beer's law (1):

$$A = \varepsilon \cdot c \cdot l \tag{1}$$

$$\Rightarrow n = c \cdot V = \frac{A}{\varepsilon \cdot l} \cdot V$$

$$\therefore \frac{\Delta n}{\Delta t} = \frac{\Delta A}{\Delta t} \cdot \frac{V}{\varepsilon \cdot l} \tag{2},$$

where ε and c are the extinction coefficient and concentration of nitrofurazone, respectively, l is the path length and V is the assay volume.

2.4.6. Disc diffusion test

Disc diffusion or Kirby-Bauer test (Bauer *et al*., 1966) was employed as an alternative method to detect nitroreductase activity in whole-cell *E. coli*. BL21-CodonPlus®(DE3)-RP competent cells were transformed (section 2.2.5) with either pET20b_TEV_*Tb*NTRΔ54 or pET20b_TEV_*Tb*NTRΔ54(Val106Ile). Cells transformed with an empty pET15b_TEV vector were used a negative control for background activity. Conversely, cells transformed with pET3a_*Ec*NfsB were used as a positive control. Expression of recombinant protein was induced in 100 ml LB media supplemented with 1-fold carbenicillin (50 μg ml^{-1}), as previously described (section 2.3.1). Following 60 h incubation at 16°C, 200 rpm, cells were harvested by centrifugation (3,800 x *g*, 10 min, 4°C). Cell pellets were resuspended in ice-cold LB media with 1-fold carbenicillin to $OD_{600} \approx 5$ (approximately 5 x 10^9 cells ml^{-1}, Wiegand *et al*., 2008) and cell suspensions (200 μl per plate) were spread evenly on duplicate LB agar plates with 1-fold carbenicillin. Plates were allowed to dry at RT for 2 h, prior to the addition of discs. Each disc contained 5 μl of 0.1 – 10 mM nitrofurazone or 1 – 100 mM nifurtimox in 100% (v/v) DMSO. Streptomycin sulphate (Merck) was used at 2 mg ml^{-1} in Milli-Q® H_2O as a control drug with a known zone of inhibition (Bauer *et al*., 1966). Alternatively, discs containing 5 μl of DMSO or H_2O were used to control for the zone of inhibition due to solvent/carrier. Following overnight incubation at 37°C, the diameter of the zone of inhibition was recorded for each disc to the nearest whole millimetre.

2.5. Cell culture of *T. brucei*

2.5.1. Cell lines and culture conditions

All *T. brucei* cell lines were derived from BSF *T. brucei* 'single-marker' S427, constitutively co-expressing T7 RNA polymerase (*T7RPOL*) and Tet repressor (*TETR*)

in tandem with the drug-resistance marker gene neomycin phosphotransferase (*NEO*; Wirtz *et al*., 1999). For convenience, these cells are hereafter referred to as wild-type *T. brucei*. Wild-type and derivative *T. brucei* cell lines were routinely subcultured every 2 – 3 days by dilution in fresh modified HMI-9T media (HMI-9 containing 56 µM 1-thioglycerol in place of 200 µM 2-mercaptoethanol) supplemented with 2.5 µg ml^{-1} G418 to maintain the expression of the T7RPOL TETR NEO expression cassette (Hirumi and Hirumi, 1989; Wyllie *et al*., 2009). Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

2.5.2. Cell density measurements

T. brucei cell numbers were determined using a CASY® cell counter Model TT (Shärfe) according to the manufacturer's guidelines. Alternatively, cells were counted manually using a Neubauer haemocytometer chamber under a light microscope (Zeiss).

2.5.3. Generation of *TbNTR* single knock-out (SKO) cell lines

To generate a *TbNTR* gene replacement cassettes, a region spanning ~600 bp either side of the *TbNTR* ORF was amplified by PCR from *T. brucei* S427 gDNA (section 2.2.1), using primers 5′UTR_-595_F and 3′UTR_+600_R (**Table 2.1**). This sequence was then used to amplify the 5′- and 3′-untranslated regions (UTRs) immediately adjacent to the start and stop codons of *TbNTR*, using primers listed in **Table 2.6**. The 470 bp long UTRs were joined in a subsequent knitted PCR via a linker BamHI-PmeI-HindIII region, introduced by the PCR primers. The endogenous NotI site present within the 3′-UTR was silenced using primers 3′UTR_mut_G89C_F and 3′UTR_mut_G89C_R, and the QuikChange Lightning site-directed mutagenesis kit (Stratagene) as per manufacturer's guidelines. The mutant 5′- 3′-UTR construct was then cloned in the NotI site of the pGEM-5Zf(+) vector (Promega), followed by the insertion of either

puromycin acetyltransferase (*PAC*) or hygromycin phosphotransferase (*HYG*) resistance genes between the BamHI and HindIII restriction sites (sections 2.2.8 and 2.2.9). The accuracy of the final SKO constructs was verified by DNA sequencing, prior to use in transfection (section 2.5.4).

2.5.4. Generation of transgenic *T. brucei* cell lines

Wild-type BSF *T. brucei* were transfected using the patented Amaxa® Nucleofector® system (Lonza), according to the improved protocol of Burkard and colleagues (Burkard *et al*., 2007). Briefly, plasmid DNA was linearised by digestion with NotI (section 2.2.8), purified by precipitation in 70% (v/v) ethanol containing 80 mM sodium acetate, and resuspended in ultrapure Milli-Q® water to 1 μg μl^{-1}. For each electroporation, 10 μg of the purified DNA were added to 4 x 10^7 wild-type cells, resuspended in 100 μl human T-cell Nucleofector® solution. Cells were transfected using programme X-001 of the Nucleofector® II electroporator and were allowed to recover overnight in 25 ml pre-warmed HMI-9T media. Following overnight incubation, 25- and 625-fold dilutions of the transfected cultures were plated in 12-well plates in the presence of the appropriate selection antibiotic (5 μg ml^{-1} phleomycin, puromycin or hygromycin). After 3 – 4 days, wells containing dividing cells were subcultured in flasks as in section 2.5.1. Subsequent experiments were performed on clonal lines (see below) derived from the primary transgenic line in the presence of selection antibiotics. In cells transfected with expression constructs, the expression of the respective exogenous protein was induced by the addition of 2.5 μg ml^{-1} tetracycline every 2 days for a minimum of 1 week.

2.5.5. Isolation of *T. brucei* clones

Clones of all *T. brucei* cell lines were prepared by limiting dilution. After the initial cell density of the relevant line was determined (see section 2.5.2), the cell suspension was

diluted to 1.5 cells ml^{-1} and plated in 96-well plates. Each well contained 200 µl suspension (equivalent to ⅓ of a cell per well). After 1 week, dividing cells were present in 15% – 30% of the wells. Usually, 5 – 10 clones were selected for further analysis.

2.5.6. Growth inhibition studies of *T. brucei*

The effective compound concentration which inhibits cell growth by 50% (EC_{50}) was used as an estimate for the *in vitro* sensitivity of *T. brucei* to various compounds. EC_{50} values were determined in 96-well plates (Greiner) as previously described (Jones *et al*., 2010a). Test compounds were prepared in DMSO and serially diluted in HMI-9T media, with the highest compound concentration in the range of 0.05 – 500 µM based on solubility (section 2.1.1) and predicted EC_{50} values. The final DMSO concentration in the assay was kept constant at 0.5 % (v/v). Wild-type or derivative BSF *T. brucei* were added, to a final concentration of 1 x 10^5 ml^{-1} for slow-growing cells or 2.5 x 10^3 ml^{-1} for fast-growing cells, in a final assay volume of 200 µl. Following a 3-day incubation (section 2.5.1), 45 µM resazurin (Sigma) was added and the plates were incubated at 37°C for further 4 – 5 h. Fluorescence due to the formation of resorufin (O'Brien *et al*., 2000) was measured at $\lambda_{excitation}$ = 528 nm and $\lambda_{emission}$ = 590 nm. Data were corrected for background fluorescence (wells with media only) and expressed as a percentage of non-treated cells. Corrected data were fitted to the following two-parameter non-linear regression, using GraFit software:

$$y = \frac{100}{1 + \left(\frac{[I]}{EC_{50}} \right)^m} \tag{3}$$

In equation (3), y is the growth percentage at inhibitor concentration $[I]$, and m is the slope factor. Measurements were repeated on at least three occasions and the data were presented as the weighted mean ± the weighted standard error of the mean (S.E.M.).

The weighted mean EC_{50} and m values were subsequently used to calculate the minimum inhibitory concentration (EC_{99}) using equation (4):

$$EC_{99} = EC_{50} \times 99^{1/m} \quad (4)$$

2.5.7. Generation of resistant *T. brucei* cell lines

A nifurtimox-resistant line was generated by subculturing wild-type *T. brucei* in the continuous presence of nifurtimox. Starting at a sublethal concentration of 1.5 μM, the drug concentration in the culture media was increased in a step-wise manner, usually by 2-fold. Cells, resistant to ≥6 μM drug, were cryopreserved before the drug concentration was increased. After a total of 140 days in culture, when trypanosomes were able to survive and grow in 50 μM nifurtimox, the resulting *T. brucei* line (designated NfxR) was cloned in the absence of nifurtimox (section 2.5.5). Two clones (NfxR1 and NfxR2) displaying the highest resistance to nifurtimox, were selected for further studies. Resistance to fexinidazole was generated in *T. brucei* is a similar manner, starting at 1.0 μM fexinidazole. Fexinidazole-resistant clones were derived from the resulting FxR line after 137 days in culture, and clones FxR1 and FxR2 were selected for further analysis.

2.5.8. Measurement of the doubling time of *T. brucei*

To determine the doubling time (t_d) of *T. brucei*, the cell density was recorded (section 2.5.2) over a period of 10 days. The logarithm of the resulting cumulative cell number (N) was plotted as a function of time (t) and the Hill slope of the linear regression,

$$m = (\log N - \log N_0)/t$$

was calculated using GraFit (N_0 is the initial cell number). The doubling time was then calculated using equation (5):

$$t_d = \frac{t}{(\log N - \log N_0)} \times \log 2 = \frac{\log 2}{m} \quad (5)$$

2.5.9. Analysis of intracellular thiols

The intracellular levels of glutathione (GSH) and trypanothione ($T[SH]_2$) were determined in wild-type and nitrodrug resistant *T. brucei*. Late-log phase trypanosomes (1 x 10^8 cells) were harvested by centrifugation (950 x *g*, 10 min, 4°C) and washed once in ice-cold PSG buffer (PBS, pH 8.0, 1.5% (w/v) glucose and 0.5 mg ml^{-1} BSA). Thiols were derivatised with monobromobimane according to a published protocol (Shim and Fairlamb, 1988). Analysis of the acid-soluble thiols by reverse phase (RP) HPLC was performed by Dr Susan Wyllie as previously described (Wyllie *et al*., 2009).

2.5.10. Pull-down of *Tb*NTR-*myc*

A tetracycline-inducible (Ti) ectopic copy of gene encoding for *Tb*NTR fused to a C-terminal c-*myc* tag (^{Ti}*TbNTR-myc*) was introduced into wild-type BSF *T. brucei* using the pLew82 vector (Wirtz *et al*., 1999), which targets the gene of interest to the ribosomal DNA locus. Clones of transgenic *T. brucei* were isolated under phleomycin selection and expression of *Tb*NTR-*myc* was induced by the addition of tetracycline to the culture media (sections 2.5.4 and 2.5.5). Expression from the ectopic gene was confirmed by a shift in nifurtimox sensitivity (section 2.5.6), observed for the induced transgenic line compared to wild-type cells. Immunoprecipitation of *Tb*NTR-*myc* was carried out based on a protocol by Dr Maria Lucia S. Güther (Güther *et al*., 2006). Wild-type and induced transgenic *T. brucei* were harvested by centrifugation (10 min, 1000 x *g*, 4°C) and washed once in PSG buffer (section 2.5.9). Trypanosomes were lysed in 20 mM Tris-HCl, pH 7.2, 2% (w/v) SDS (1 ml per 10^9 cells). Samples were vortexed for 3 min to shear gDNA and reduce viscosity, and then boiled for 10 min. Following equilibration to RT, samples were diluted to 0.1% (w/v) SDS in 20 mM Tris, pH 7.2, 1% Triton® X-100 and immunoprecipitated with anti-Myc Tag mAb (clone 4A6, Upstate/Millipore; 1 µg per 1 x 10^8 cells) overnight at 4°C with gentle agitation.

The *Tb*NTR-*myc*-mAb complex was pulled-down using Pierce® Protein G Plus Agarose (Thermo Scientific) according to the manufacturer's guidelines, at a ratio of 5 µl beads per 1 µg mAb. Washed beads were boiled for 10 min in 2 x Laemmli sample buffer with 10 mM DTT, resolved by SDS-PAGE (section 2.4.2), and analysed by Western blotting onto a Sequi-blot PVDF membrane (Bio-Rad) as previously described (section 2.4.3).

2.6. Animal studies

The work under this section was carried out following review by the Ethical Review Committee of the University of Dundee and performed under the Animals (Scientific Procedures) Act 1986 in accordance with the European Committee Council Directive (86/609/EEC).

All animal work during the generation of anti-*Tb*NTR sera (section 2.6.1), as well as the study on virulence (section 2.6.2) and susceptibility to nifurtimox (section 2.6.3), was carried out by Dr Susan Wyllie. The study on fexinidazole susceptibility (section 2.6.4) and all pharmacokinetic analysis of fexinidazole and its metabolites (section 2.6.5) were performed by Dr Kevin D. Read and the DMPK group, University of Dundee.

2.6.1. Generation of anti-*Tb*NTR sera

Polyclonal anti-*Tb*NTR antisera were raised in male Wistar rats using recombinant His_6-*Tb*$NTR_{\Delta 7}$, purified under denaturing conditions from inclusion bodies (section 2.3.10). To remove traces of guanidinium-HCl, purified protein was diluted and washed once in PBS. The resulting protein precipitate was resuspended in fresh PBS to 1 mg ml^{-1}, emulsified in Freund's (in)complete adjuvant (Sigma-Aldrich®) and used to raise antisera exactly as described (Greig *et al*., 2009). Blood was harvested from the animals

5 weeks after the initial inoculation and clotted at 37°C for 1 h. Cells were discarded following centrifugation (3,000 x *g*, 15 min, 4°C) and the resulting antisera were flash-frozen and stored at -20°C in the presence of 0.05% (w/v) sodium azide.

2.6.2. Virulence studies

The ability of NfxR1 and NfxR2 BSF trypanosomes (section 2.5.7) to establish an *in vivo* infection was studied in an animal model. Sets of 5 NMRI mice were infected via a single intraperitoneal (i.p.) injection of 1 x 10^4 cells (wild-type, NxfR1 or NfxR2) in 0.2 ml HMI-9T media. Animals were monitored daily for clinical signs of infection and wet tail-blood smears were taken for microscopic examination, in order to determine the levels of parasitaemia (Sienkiewicz *et al*., 2008). When parasitaemia levels reached or exceeded 1 x 10^8 ml^{-1}, the animals were humanely killed, since it was previously established that these animals would succumb to an over-whelming infection within 24 h. Results are reported as the mean survival time ± standard deviation (S.D.).

2.6.3. *In vivo* susceptibility to nifurtimox

To determine whether NfxR1 trypanosomes maintain their nifurtimox resistance *in vivo*, the following experiment was conducted. Infection with wild-type and NfxR1 parasites was established in groups of 5 NMRI mice as in section 2.6.2. Twenty-four hours following infection, wild-type- and NfxR1-infected mice were either dosed with nifurtimox (100 mg kg^{-1} body weight, based on Fairlamb *et al*., 1992) or left untreated. Nifurtimox was administered daily for a total of four days via i.p. injection, using 10% (v/v) DMSO in peanut oil as a carrier. Parasitaemia was monitored for a maximum of 30 days, when the experiment was terminated. In addition to animals with high levels of parasitaemia, animals showing signs of acute drug toxicity were also euthanized. The data are presented as a Kaplan-Meier survival plot.

2.6.4. *In vivo* susceptibility to fexinidazole

The susceptibility of NfxR1 and wild-type trypanosomes to fexinidazole was studied in a mouse model. Experiments were performed as described for nifurtimox (section 2.6.3), with the following modifications. Fexinidazole was administered orally and at doses of 25 – 200 mg kg^{-1}. Parasitaemia was monitored for up to 60 days post infection, and animals that survived for the entire duration of the experiment were defined as "cured". The fraction of cured animals was plotted against the respective dose of fexinidazole using GraFit software. The effective dose, required to elicit 50% cure (ED_{50}) was calculated using equation (6):

$$f_c = \frac{1}{1+\left(\frac{ED_{50}}{D}\right)^m} \tag{6}$$

where f_c is the fraction of cured animals, D is the administered dose, and m is the Hill slope. The ED_{99} was derived from the above equation as:

$$ED_{99} = ED_{50} \times 99^{1/m} \tag{7}$$

2.6.5. Fexinidazole exposure in mice after a single oral dose

NMRI mice (n = 3) were dosed with a single oral dose of 200 mg ml^{-1} fexinidazole in 10% (v/v) DMSO in peanut oil. At defined time points after dosing, 10 µl blood samples were withdrawn from the tail vein of each animal, lysed in 20 µl deionised water (Milli-Q®) into Micronic tubes (Micronic BV) and stored at -80°C prior to analysis. Following extraction with acetonitrile in the presence of an undisclosed internal standard, the samples were analysed by ultraperformance liquid chromatography (UPLC)-MS-MS, using Waters Acquity UPLC® system and a Quattro Premier™ XE mass spectrometer (Waters, UK) as described (Sokolova *et al*., 2010). The levels of fexinidazole, fexinidazole sulfoxide and fexinidazole sulfone in these samples

were determined from calibration curves, that were prepared by analysing blood samples with known amounts of fexinidazole (in the range of 1 – 1,000 ng ml^{-1}) and its metabolites (200 – 15,000 ng ml^{-1}).

Chapter 3 - Expression and activity studies of recombinant *Tb*NTR

3.1. Isolation and characterisation of *NTR* from *T. brucei* S427

3.1.1. DNA sequencing analysis

Search of the trypanosomatid genome database (http://www.genedb.org) revealed two sequences of a putative nitroreductase: one in the genome reference strain *T. brucei* TREU927 GUTat 10.1 (Tb927.7.7230 or *TbNTR*-927) and another one in *T. b. gambiense* (Tbg972.7.8530 or *TbgNTR*). The two sequences were almost identical and had only two differences at the nucleotide level and one at the amino acid level (Ile57 replaced by Thr in *Tbg*NTR). To isolate the sequence of *NTR* from the laboratory strain *T. brucei* S427 (designated *TbNTR*-427), amplification and sequencing primers were designed based on the *TbNTR*-927 sequence. The ORF of *TbNTR* and the adjacent 5′- and 3′-UTRs (~600 bp each) were amplified by PCR from *T. brucei* S427 gDNA and the resulting 2.16 kb fragment (**Figure 3.1 A**) was cloned into the pCR®-Blunt II-TOPO® vector. Initial sequencing analysis of clones derived from 3 independent PCRs revealed the presence of single nucleotide polymorphisms (SNPs) in the ORF. To confirm that these were not caused by PCR or sequencing error, *TbNTR*-427 ORF was amplified in a second set of three PCRs, cloned and re-sequenced. A total of four SNP positions were identified (**Figure 3.1 B**), two of which were either T or C (**Y**) and the other two were either G or A (**R**). Although the polymorphism in codon 31 (aa**Y** = Asn) was silent, it is worth noting that the same codon is aat in *TbNTR*-927 and aac in *TbgNTR*. The remaining three SNPs, which resulted in amino acid changes, were in codons 57 (atc = Ile or acc = Thr), 69 and 261 (both gat = Asp or aat = Asn). Each couple of polymorphic nucleotides was not equally represented in the sequenced clones. For example, aat31 was present in 2 out of 6 clones, whereas aac31 was present in the remaining 4 clones. As a result, the sequence variant of *TbNTR*-427 that was observed most frequently (Thr57/Asp69/Asn261) was selected and used in all subsequent cloning and expression studies, and is hereafter referred to as *TbNTR* for simplicity.

3.1.2. Determination of the *NTR* gene copy number in *T. brucei* S427

To investigate whether the observed sequence variations in *TbNTR*-427 were caused by the presence of multiple gene copies, a Southern hybridisation analysis was performed. Restriction enzymes, which cut outside (KpnI and HindIII) or within (BamHI, PstI and SalI) the *TbNTR* ORF (**Figure 3.2 A**) were identified in the genome database and used to digest *T. brucei* S427 gDNA. Following hybridisation with a digoxigenin-dUTP-labelled *TbNTR* probe, single restriction fragments were detected in the KpnI and HindIII digests (**Figure 3.2 B**), whereas two fragments were visualised in DNA digested with the endonucleases which cut within the *TbNTR* ORF. Although PstI is predicted to cut *TbNTR* twice and produce three fragments, the shortest of those would be only 146 bp and would not be visible on the blot. Collectively, these results conform to the predicted patterns for a single gene copy (per haploid genome) of *TbNTR*. Therefore, multiple copy number cannot account for the observed sequence variations *TbNTR*-427, which leaves two alternative explanations. Either there is an allelic variation at the *TbNTR* gene locus, or the *T. brucei* culture, from which the gDNA was extracted, is no longer clonal.

3.2. Expression, purification and activity studies of *Ec*NfsB

The host *E. coli* nitroreductase with highest similarity to *Tb*NTR (*Ec*NfsB, 27% similarity) was used as a positive control for expression trials and activity studies. *Ec*NfsB was purified by sequential hydrophobic interactions and anion exchange chromatography to >95% purity (**Figure 3.3 A**), with a total protein yield of 12 – 13 mg per 1 L culture. The specific activity of purified *Ec*NfsB with nitrofurazone was found to increase from 4 U mg^{-1} to 16 ± 1 U mg^{-1} upon addition of 10 µM FMN either to the assay mix (**Figure 3.3 B**) or to the purified enzyme stock (*Ec*NfsB ≈ 20 µM). The specific activity obtained in the presence of FMN is in close agreement with the

reported literature value of 13 U mg^{-1} for FMN-bound *Ec*NfsB (Zenno *et al*., 1996). The purified enzyme retains >80% of its activity after storage for 1 year at -80°C in the presence of 20% (v/v) glycerol and 10 μM FMN.

3.3. Expression and partial purification of full-length *Tb*NTR

3.3.1. Optimisation of conditions for the expression of V5-*Tb*NTR

Initial attempts to express and purify recombinant *Tb*NTR were based on protocols available at the time for purification of the protozoal nitroreductase from *Giardia lamblia Gl*NR1 (Muller *et al*., 2007). To facilitate purification, full-length *TbNTR* was cloned into the pET151/D-TOPO® vector for expression of the V5-*Tb*NTR fusion protein, which features an N-terminal hexahistidine (His_6) tag, followed by the V5 epitope and a TEV recognition site (**Table 2.3**). Pilot expression was carried out in BL21 Star™(DE3), which expressed recombinant NTR at very low levels, detectable only by Western blot analysis with monoclonal anti-His antibodies (**Figure 3.4 A**). Furthermore, the presence V5-*Tb*NTR in the supernatant was found to be caused by contamination with insoluble material from the very soft pellet obtained following centrifugation. Since V5-*Tb*NTR could be expressed neither at high levels nor as a soluble protein, several alternative strategies (**Table 2.4**) were attempted in order to improve expression and solubility of the recombinant protein.

Optimisation of the expression strain was suggested by sequence analysis of *TbNTR*-427 (**Figure 3.1 B**), which revealed the presence of at least 21 codons not commonly found in the *E. coli* host (Zhang *et al*., 1991). Although *E. coli* CodonPlus®(DE3)-RP and -RIL strains, engineered to produce tRNAs for such rare codons, resulted in much higher levels of recombinant protein expression (**Figure 3.4 B**), V5-*Tb*NTR expressed by these strains remained completely insoluble.

The best results were achieved using ArcticExpress™(DE3)RP cells (**Figure 3.4 C**), which not only combine increased protein levels (via additional Arg and Pro tRNAs) with reduced expression rates (at 12°C), but also have an improved ability to process heterologous proteins at low temperature, conferred by co-expression of chaperonins Cpn60 and Cpn10 from *Oleispira antarctica* (Ferrer *et al*., 2004; Ferrer *et al*., 2003). To date, these were the only cells among those tested that expressed apparently soluble V5-*Tb*NTR, albeit only a small proportion of the total recombinant protein.

3.3.2. Partial purification of V5-*Tb*NTR

Following expression of partly soluble recombinant *Tb*NTR in ArcticExpress™(DE3) RP, attempts were undertaken to purify V5-*Tb*NTR. However, the method of choice – Ni^{2+} affinity chromatography, proved unsuccessful for V5-*Tb*NTR purification under the test conditions (**Figure 3.5**). Part of the protein did not bind to the column (lane 2), and the remainder was found associated with numerous contaminating bands in both peak 1 (lane 3) and peak 2 (lane 4) following elution with an imidazole gradient. In addition, contaminants, including chaperonin *Oa*Cpn60, were much more abundant than the protein of interest after chromatography.

Bearing in mind the poor yield and recovery, further experiments were aimed at improving the ratio of recombinant *Tb*NTR found in the supernatant and pellet fractions. To explore the effects of buffer composition, pH and ionic strength, several lysis buffers were tested (**Table 2.4**, condition 4). Best results were achieved in 50 mM HEPES, pH 7.5, in the absence of 0.25 M NaBr (**Figure 3.6 A**), suggesting that high ionic strength may decrease solubility of V5-*Tb*NTR. Similar results were obtained with 50 mM Tris-HCl, pH 8.5, without NaBr (not shown). More recombinant protein was found to sediment with greater centrifugal force (**Figure 3.6 B**), which prompted the

addition of 10% (v/v) glycerol to the final optimised lysis buffer. Other factors, such as change of expression media, freezing of the cell pellet prior to lysis, and the addition of FMN or Triton® X-100 to the lysis buffer, did not affect solubility of V5-*Tb*NTR (data not shown).

Under the optimised cell lysis conditions (50 mM HEPES, pH 7.5, 10% (v/v) glycerol), V5-*Tb*NTR did not bind to the affinity column (**Figure 3.7** lane 3). Nonetheless, this step was used to remove contaminating histidine-rich host proteins. Surprisingly, recombinant *Tb*NTR was also found not to bind to a cation exchange column (HiTrap™ SP FF, not shown) at pH 7.5, despite a predicted pI of 9.84 for *Tb*NTR and 9.67 for the tagged V5-*Tb*NTR (ProtParam tool on the ExPASy Proteomics Server, http://www.expasy.org/). Further purification was achieved by two sequential anion exchange steps under the same conditions, which removed a considerable amount of the major contaminant *Oa*Cpn60 (**Figure 3.7** lanes 4 and 5). The remaining contaminants (visible in lane 6) could not be resolved by gel filtration on a HiLoad™ 16/60 Superdex™ 200, and co-eluted with V5-*Tb*NTR as a single peak at the void volume (V_0 = 600 kDa) of the column (lane 7). Mass-fingerprinting analysis of SDS-PAGE samples positively identified the two major bands as recombinant *Tb*NTR (at ~39 kDa) and *Oa*Cpn60 (at ~60 kDa), and confirmed that lower MW bands were mostly contaminants, rather than NTR degradation products. Although host *E. coli* nitroreductases (NfsA, 26.8 kDa and NfsB, 23.9 kDa) were not found in this proteomics analysis, which is important for future activity studies, the vast majority of the remaining contaminants were chaperonins, membrane transporters, electron transport components and ribosomal proteins. Together these results indicated that V5-*Tb*NTR was either associated with or entrapped in membrane vesicles. Consequently, purification of recombinant full-length *Tb*NTR was not pursued any further.

3.4. Truncated forms of *Tb*NTR

3.4.1. Truncations based on bioinformatics predictions: *Tb*NTR$_{\Delta 57}$ and *Tb*NTR$_{\Delta 85}$

ClustalW (Larkin *et al*., 2007) multiple sequence alignment of *Tb*NTR and other protozoal and bacterial nitroreductases (**Figure 3.8**) revealed a distinct stretch of 70 – 80 amino acids with little sequence conservation at the N-terminus of the kinetoplastid sequences, which is absent from the bacterial enzymes. By contrast, in the remaining C-terminal sequence, kinetoplastid NTRs share >55% identity and >70% similarity. This C-terminal domain is referred to as the nitroreductase domain, since it contains all key conserved residues (highlighted in bold), shown to hydrogen bond with the FMN co-factor in bacteria (Parkinson *et al*., 2000). In the genome database, part of the variable N-terminal region is annotated as a mitochondrial targeting sequence for three of the four putative *Leishmania* NTRs (from *L. major*, *L. infantum* and *L. braziliensis*). To confirm whether this is the case in *T. brucei*, the protein sequences of *Tb*NTR-927 and -427 were analysed using MitoProt II (version v1.101, Claros and Vincens, 1996). Both proteins were predicted as targeted to the mitochondria with a probability of p ~ 0.88, and a putative cleavage site was assigned after amino acid 57, regardless of the sequence variant (Thr57 or Ile57). Based on these results, two truncated forms of *Tb*NTR were engineered. *Tb*NTR$_{\Delta 57}$ lacks the sequence predicted to be cleaved after import into the mitochondrion, whereas *Tb*NTR$_{\Delta 85}$ lacks the entire variable N-terminal region and has a start site aligned with the first amino acid after the initiator methionine of *Ec*NfsB.

The expression profiles of both V5-*Tb*NTR$_{\Delta 57}$ and V5-*Tb*NTR$_{\Delta 85}$ were very similar to that of the full-length recombinant protein. Under the initial lysis conditions, V5-*Tb*NTR$_{\Delta 57}$ appeared completely insoluble (**Figure 3.9 A**), and under the optimised lysis conditions, partly soluble V5-*Tb*NTR$_{\Delta 85}$ could not be purified by affinity chromatography (**Figure 3.9 B**), as was previously observed for the full-length

construct. These results indicated that removing potential mitochondrial targeting sequences from *Tb*NTR alone may not be sufficient to improve solubility or amenability to purification of the recombinant protein.

To address the poor retention of recombinant *Tb*NTR by the affinity resin, full length and truncated forms of the protein were expressed with a C-terminal, TEV-cleavable His_6 tag, using the modified pET20b_TEV expression vector (**Table 2.3**). However, results for all three forms of *Tb*NTR-His_6 were almost identical to those for the N-terminally tagged constructs. Recombinant *Tb*NTR-His_6 (full-length and truncations) was predominantly insoluble (>95% by Western), and *Tb*$NTR_{\Delta 85}$-His_6 failed to bind to a HisTrap™ HP affinity column (data not shown). Therefore, positioning of the His_6 tag at the C-terminus did not improve interaction with the affinity resin.

During the course of the above experiments, purification and partial characterisation of NTR from *T. cruzi* (Wilkinson *et al*., 2008) and *T. brucei* (Hall *et al*., 2010) was reported. To match the form of *Tc*NTR described in the former study, a slightly different truncated form of *Tb*NTR (lacking the first 83, not 85 amino acids) was cloned into the pTrcHis C vector with an N-terminal, enterokinase-cleavable, His_6 tag (**Table 2.3**). Several unsuccessful attempts (**Figure 3.9 C** and **D**) were made to purify His_6-EK-*Tb*$NTR_{\Delta 83}$, hampered by lack of detail in the authors' experimental protocol. Subsequent conversations with Wilkinson and colleagues established that CHAPS, sucrose and high salt were all used in the lysis buffer, in order to retain the recombinant protein in solution and facilitate interaction with affinity columns. However, these specific conditions were not tested with the current construct (His_6-EK-*Tb*$NTR_{\Delta 83}$) due to new information that arose throughout the course of the investigation (see section 3.4.3).

3.4.2. Expression of *Tb*NTR$_{\Delta 83}$ with large soluble tags

In order to improve the solubility, and potentially amenability to purification, of recombinant *Tb*NTR, the new truncated form (Δ83) was fused to various *E. coli* proteins, which could serve as molecular anchors and help retain the passenger protein in solution (Terpe, 2003). Initial tests were carried out using elongation factor Ts (Tsf, 30.5 kDa) as a fusion partner, which had been shown to enhance the solubility of the putatively mitochondrial DHFR-TS from *T. brucei* (Marc W. Gibson, PhD thesis, University of Dundee, 2009). Although the recombinant Tsf-TEV-*Tb*NTR$_{\Delta 83}$ protein fusion was highly soluble (at 50,000 x *g*, 1 h), it was only partially retained on a HisTrap™ affinity column (data not shown). Furthermore, the Tsf-TEV tag was not susceptible to TEV protease cleavage at near optimal conditions (25 – 37°C) and high protease to substrate ratios (up to 1:5 w/w). Similar difficulties in purification by Ni^{2+} affinity chromatography and removal of the tag had also been observed by Dr Gibson for the analogous Tsf-TEV-*Tb*DHFR-TS construct, suggesting that steric occlusion by the large tag was the most likely cause. However, in contrast to Tsf-TEV-*Tb*DHFR-TS, which was enzymatically active (Gibson, PhD thesis, 2009), no nitroreductase activity could be detected for recombinant Tsf-TEV-*Tb*NTR$_{\Delta 83}$.

In a second approach, the transcription termination anti-termination factor N-utilising substance A (NusA, 55 kDa) was used as a soluble anchor, followed by affinity His_6 and S tags, and thrombin and enterokinase recognition motifs upstream of *Tb*NTR$_{\Delta 83}$ (**Figure 3.10**). As expected, the resulting recombinant NusA-*Tb*NTR$_{\Delta 83}$ protein fusion was expressed at high levels in a soluble form. However, the partially purified recombinant protein (lane 3) was not enzymatically active. To test whether removal of the tag would lead to gain of nitroreductase activity, NusA-*Tb*NTR$_{\Delta 83}$ was digested using thrombin protease. Proteolytic cleavage at 37°C resulted in the formation of lower MW products (lane 4), possibly due to recognition of the secondary thrombin

sites present in the *Tb*NTR sequence (**Figure 3.10 B**). Although at lower temperatures (4°C) the formation of secondary cleavage products was reduced, the enzymatic digest was incomplete (lanes 5 and 6). Furthermore, *Tb*NTR$_{\Delta 83}$ cleaved at 4°C was found to co-elute with undigested NusA-*Tb*NTR$_{\Delta 83}$ (lane 7), possibly through heterodimer formation. Therefore, complete cleavage of the tag appeared to be a prerequisite for obtaining pure cleaved *Tb*NTR$_{\Delta 83}$. However, attempts to cleave NusA-*Tb*NTR$_{\Delta 83}$ with other proteases were unsuccessful. Digestion with enterokinase was highly inefficient even at the highest recommended ratio of 5 U per 100 µg substrate (data not shown). In a similar way, digestion with TEV protease of partly purified NusA-TEV-*Tb*NTR$_{\Delta 83}$ (thrombin site replace with a TEV site) resulted in only partial cleavage of the tag (not shown). Given that both tagged and cleaved unpurified *Tb*NTR$_{\Delta 83}$ were not enzymatically active (preliminary data, not shown), and that pure cleaved *Tb*NTR$_{\Delta 83}$ could not be obtained, this truncated form of *Tb*NTR was not investigated further.

3.4.3. Design of alternative *Tb*NTR truncations

During the course of the above investigations, an analysis of the mitochondrial proteome of procyclic *T. brucei* was published (Panigrahi *et al*., 2009) confirming the mitochondrial localisation of *Tb*NTR. The above study identified six peptides derived from *Tb*NTR (highlighted in red in **Figure 3.11**), the first of which started at Ser55. From these data, it was evident that the truncated versions of *Tb*NTR, which have been studied so far (Δ57 and Δ83/85), were apparently missing parts of the mature protein, after import into the mitochondrion. Consequently, seven new truncated forms of *Tb*NTR were designed so that their starting sites were upstream of Ser55 (**Figure 3.11**). The first residue for each truncation was selected taking into account secondary structures, such as α-helices and β-strands predicted by Jpred 3 (Cole *et al*., 2008), and the tertiary structure assigned by GlobPlot™, which predicted a globular NTR domain

for residues 47 to 296 (Linding *et al*., 2003). An additional consideration was given to the N-end rule, where the residue immediately following the initiator methionine can confer either stability or instability to the protein by affecting its half-life (Tobias *et al*., 1991).

3.4.4. Activity studies in truncated forms of *Tb*NTR

To find out which of the newly designed *Tb*NTR truncations can be expressed in an enzymatically active form in *E. coli*, the nitroreductase activity of clarified bacterial lysates expressing each truncated form was compared to the background activity in cells transformed with the blank vector (**Figure 3.12**). To avoid any interference on recombinant protein activity, all *Tb*NTR forms were expressed without affinity/solubilisation tags. A control lysate of cells expressing *Ec*NfsB had almost 70 times higher specific activity compared to the empty vector control lysate (800 ± 120 mU mg^{-1} and 12 ± 3 mU mg^{-1}, respectively, p = 0.001 by Student's *t*-test). The only other lysate, which was found to have a significantly high activity above background (2.3-fold, p = 0.01), was of cells expressing $TbNTR_{\Delta 54}$ (27 ± 5 mU mg^{-1}). Although the specific activities of different lysates cannot be compared directly to each other because of huge variations in the expression levels of recombinant protein (**Figure 3.12**), the presence of activity above background indicates that at least some of the respective recombinant protein is expressed in an active form. On the other hand, lack of activity above background does not necessarily mean that a given truncated form of the protein is intrinsically inactive, as this protein may not be expressed in a soluble form at a sufficiently high amount for detection.

3.4.5. Positional effect of the affinity tag on *Tb*NTR$_{\Delta 54}$

The only truncation of *Tb*NTR that was expressed in an enzymatically active form in *E. coli*, *Tb*NTR$_{\Delta 54}$, was present only at low levels, which were deemed insufficient for protein purification by classical chromatography. Hence, the use of an affinity tag was necessary to facilitate purification. To test the effect of a His$_6$ affinity tag on *Tb*NTR$_{\Delta 54}$, the recombinant protein was expressed with either an N-terminal or a C-terminal TEV-cleavable His$_6$ tag (**Table 2.3**), and compared to the non-tagged form (**Figure 3.13**). Significant nitroreductase activity above background was observed only in lysates expressing non-tagged (5.7-fold, $p < 0.001$) or C-terminally tagged (8-fold, $p < 0.001$) *Tb*NTR $_{\Delta 54}$. This data suggested that a tag at the N-terminus may lead to loss of activity in *Tb*NTR$_{\Delta 54}$, whereas a C-terminal tag is better tolerated.

Estimated data for the activity of *Tb*NTR$_{\Delta 54}$ and *Tb*NTR$_{\Delta 54}$-His$_6$ were obtained by subtraction of the endogenous nitroreductase activity (8.8 ± 0.1 mU mg^{-1} in vector-only cells). The resulting background corrected specific activity value was 37 ± 2.5 mU per mg lysate for non-tagged *Tb*NTR$_{\Delta 54}$, and 57 ± 2 mU per mg lysate for *Tb*NTR$_{\Delta 54}$-His$_6$. After overnight storage at 4°C, those values decreased to 28 ± 4 mU mg^{-1} lysate (~76%) and 46 ± 3 mU mg^{-1} lysate (~80%) for non-tagged and tagged *Tb*NTR$_{\Delta 54}$, respectively, possibly due to proteolytic degradation. Almost identical values were obtained using nifurtimox as a substrate after overnight storage (23 ± 1 mU mg^{-1} lysate for *Tb*NTR$_{\Delta 54}$ and 44 ± 3 mU mg^{-1} lysate for *Tb*NTR$_{\Delta 54}$-His$_6$), suggesting that the enzymes uses both nitrodrugs as substrates with equal efficiency.

3.4.6. Activity studies, partial purification and solubilisation of *Tb*NTR$_{\Delta 54}$-His$_6$

Once it was established that C-terminally tagged *Tb*NTR$_{\Delta 54}$ could be expressed in an enzymatically active form in *E. coli*, purification by affinity chromatography of recombinant *Tb*NTR$_{\Delta 54}$-His$_6$ was attempted. However, the majority of *Tb*NTR$_{\Delta 54}$-His$_6$

was found in the flow-though fraction (**Figure 3.14**, lane 4), together with 87% of both the protein mass and the nitroreductase activity that were applied to the HisTrap™ column. Better results were obtained using anion exchange chromatography (at pH 7.0), which was previously shown to remove the minor host nitroreductase *Ec*NfsB (pI = 5.8, ProtParam; section 3.2). As expected, *Tb*NTR$_{\Delta 54}$-His$_6$ did not bind to the anion exchange column (lane 5; predicted pI = 9.2, ProtParam), but only ~50% of the protein mass and as much as 75% of the activity were recovered in the flow though. Consequently, a 1.5-fold increase in the specific activity of the *Tb*NTR$_{\Delta 54}$-His$_6$-containing fraction was observed (from 50 ± 4 mU mg^{-1} to 78 ± 8 mU mg^{-1}). Other chromatographic methods, including cation exchange chromatography (HiTrap™ SP FF, lane 6), Co^{2+} affinity chromatography (lane 7) and nucleotide-mimicking Blue Sepharose™ chromatography (lane 8), failed to bind recombinant *Tb*NTR$_{\Delta 54}$-His$_6$. Similar to N-terminally tagged full-length *Tb*NTR (V5-*Tb*NTR, **Figure 3.7**), *Tb*NTR$_{\Delta 54}$-His$_6$ was apparently not amenable to purification. Furthermore, *Tb*NTR$_{\Delta 54}$-His$_6$ was found in the void volume of a Superdex™ 75 gel filtration column (lane 10, V_0 = 75 kDa), co-eluting with many of the chaperonins, membrane transporters and electron transport components that were previously identified after gel filtration of V5-*Tb*NTR. Collectively, these results indicate that recombinant *Tb*NTR$_{\Delta 54}$-His$_6$ may also be associated with/entrapped in membrane vesicles, as suggested for V5-*Tb*NTR.

To establish whether *Tb*NTR$_{\Delta 54}$-His$_6$ is truly soluble or membrane-associated, clarified lysates of cells expressing *Tb*NTR$_{\Delta 54}$-His$_6$ (**Figure 3.15**, lane 2) were subjected to centrifugation at 100,000 x *g* for 1 h. At this high centrifugal force, *Tb*NTR$_{\Delta 54}$-His$_6$ was found almost exclusively in the pellet fraction (lane 5), which was associated with ~50% of the nitroreductase activity recorded before centrifugation. By contrast, only ~20% of the initial nitroreductase activity was recovered in the supernatant, and half of this activity was shown to be caused by endogenous *E. coli* enzymes. In sharp contrast,

in control cell lysates (vector-only), >99% of the nitroreductase activity was recovered in the high-force supernatant. Treatment of the 100,000 x *g* pellet with 0.1% (w/v) n-dodecyl-β-D-maltoside (DDM; cmc = 9.2 % w/v, Privé, 2007) marginally increased the specific activity of the sample (by 10%), but failed to solubilise recombinant *Tb*NTR$_{\Delta 54}$-His$_6$. An alternative treatment suggested by Dr S.R. Wilkinson (section 3.4.1) involved cell lysis in the presence of 0.5 M NaCl, 40% (w/v) sucrose and 1% (w/v) CHAPS. However, this buffer also failed to prevent *Tb*NTR$_{\Delta 54}$-His$_6$ from sedimentation at 100,000 x *g* for 1 h (note that the centrifugation speed used by Wilkinson and colleagues was 16,000 x *g*). Unsurprisingly, affinity purification in the presence of either DDM (see **Figure 3.18 C**) or NaCl/sucrose/CHAPS-buffer was unsuccessful. Collectively, these findings indicate that *Tb*NTR$_{\Delta 54}$-His$_6$ is not soluble even under the conditions reported to work by Wilkinson and colleagues.

To determine whether *Tb*NTR$_{\Delta 54}$-His$_6$ is an integral membrane protein or is associated at the periphery, clarified cell lysates were centrifuged at 100,000 x *g* for 1 h in the presence of detergents (CHAPS, 5-cyclohexylpentyl β-D-maltoside, n-decyl-β-D-maltoside, DDM, n-octyl-β-D-glucoside, n-dodecylphosphocholine and SDS, 1% (w/v) each), high salt (1 M NaCl), urea (4 M) or alkali (0.2 M Na_2CO_3). From the tested detergents, only n-dodecylphosphocholine and the control SDS were able to solubilise *Tb*NTR$_{\Delta 54}$-His$_6$ (**Figure 3.16**). However, nitroreductase activity was completely lost upon SDS treatment and substantially reduced (to ~7%) in the presence of n-dodecylphosphocholine (from 42 ± 4 mU mg^{-1} to 3 ± 0.3 mU mg^{-1}, corrected for endogenous activity). By contrast, in vector-only cell lysates the nitroreductase activity was reduced to ~34% (from 8 ± 1.3 mU mg^{-1} to 2.7 ± 0.1 mU mg^{-1}). Although recovery of enzyme activity in the high-force supernatant treated with n-dodecylphosphocholine was >99%, compared to 9% recovered in non-treated control samples (in buffer with 150 mM NaCl), the specific activity of the two supernatants was almost identical (5.5 ±

0.4 mU mg^{-1} for treated and 4.3 ± 1.1 mU mg^{-1} for non-treated samples, background corrected). Other detergents, including CHAPS, and high salt (both used by Wilkinson) were ineffective for solubilisation of *Tb*$NTR_{\Delta 54}$-His_6. Urea and alkali, which are known to solubilise only peripheral but not integral membrane proteins (De Leeuw *et al*., 2001), resulted in partial retention of the recombinant protein in the 100,000 x *g* supernatant. However, samples treated with either of these reagents lost their nitroreductase activity completely. Collectively, these results suggesting that *Tb*$NTR_{\Delta 54}$-His_6 may be membrane associated (rather than integral to the membrane) and that solubilisation of *Tb*$NTR_{\Delta 54}$-His_6 with the tested reagents results in loss of enzymatic activity.

3.5. Cell-free expression of *Tb*$NTR_{\Delta 54}$-His_6

To test if it is possible to obtain active *Tb*$NTR_{\Delta 54}$ in the absence of biological membranes, recombinant *Tb*$NTR_{\Delta 54}$-His_6 was expressed in a wheat germ cell-free extract, using linearised pET20b_TEV_*Tb*NTRΔ54 vector (**Figure 3.17 A**). Recombinant *Ec*NfsB was expressed in parallel as a positive control. Although the nitroreductase activity in *Ec*NfsB-expressing extract was at the limit of detection, addition of 5 – 20 µM FMN was found to increase the specific activity of those extracts almost 10-fold (from ~2.5 µU $µl^{-1}$ extract to 23 µU $µl^{-1}$; **Figure 3.17 B**). Extrapolating from the specific activity of recombinant *Ec*NfsB (section 3.2), it was calculated that ~1.4 ng *Ec*NfsB per µl extract was expressed as an enzymatically active protein. By contrast, no nitroreductase activity above background could be detected in *Tb*$NTR_{\Delta 54}$-His_6-expressing extracts, despite the presence of FMN. Expression of trace amounts of *Tb*$NTR_{\Delta 54}$-His_6 was verified by Western blot analysis (**Figure 3.17 C**) and the concentration of the recombinant protein was estimated at ~6.5 ng $µl^{-1}$, based on the

band intensity of a similar His-tagged construct of a known amount (His_6-*Tb*$NTR_{\Delta 7}$; see section 3.6.1 and **Figure 3.18 B**).

The estimated concentration of *Tb*$NTR_{\Delta 54}$-His_6 is within the expected range for this particular system (1 – 10 ng μl^{-1}) and studies with the positive control (*Ec*NfsB) have shown that it is possible to detect nitroreductase activity at such low enzyme concentrations, with no apparent inhibition from the cell-free system. It may be tempting to speculate that association of *Tb*$NTR_{\Delta 54}$-His_6 with biological membranes may be required for its enzyme activity, as previous studies demonstrated that solubilisation of the recombinant protein with detergents resulted in loss of activity. However, absence of detectable activity may be due to incorrect folding of the recombinant protein in the cell-free system. This notion is partially supported by the fact that *Tb*$NTR_{\Delta 54}$-His_6 could not be purified from cell-free extracts by affinity chromatography (**Figure 3.17 C**, lane 4), although it is not known whether this cell-free system is permissive for Ni^{2+}-affinity binding. Furthermore, the expression temperature (30°C) optimised for this system may be too high, since expression of *Tb*$NTR_{\Delta 54}$-His_6 in bacteria at 37°C resulted in an inactive recombinant protein, whereas *Tb*$NTR_{\Delta 54}$-His_6 expressed at 16°C was enzymatically active (not shown).

3.6. In pursuit of the true start site of mature *Tb*NTR

In order to detect the first residue of mature *Tb*NTR, two strategies were undertaken for the isolation of the processed protein from *T. brucei* by immunoprecipitation. The first strategy was dependent on the use of anti-*Tb*NTR antibodies raised in house, whereas the second one involved expression of *Tb*NTR with an epitope against which antibodies are commercially available.

3.6.1. Production of anti-*Tb*NTR serum in rats

It was necessary to obtain purified recombinant *Tb*NTR to raise polyclonal antisera in rats. Given that purification of the enzymatically active form of the protein (*Tb*NTR$_{\Delta 54}$-His$_6$) proved difficult under native conditions, efforts were directed to other forms of *Tb*NTR. Inactive recombinant His$_6$-*Tb*NTR$_{\Delta 7}$ was purified to >90% under denaturing condition (**Figure 3.18 A**) and the identity of the purified protein was confirmed by mass fingerprinting. Contaminants migrating at both higher and lower MW than the recombinant protein were also identified as *Tb*NTR, suggesting the formation of dimers and degradation products during the purification process. Antisera raised against purified His$_6$-*Tb*NTR$_{\Delta 7}$ in collaboration with Dr Susan Wyllie, University of Dundee, were able to recognise both recombinant His$_6$-*Tb*NTR$_{\Delta 7}$ and *Tb*NTR$_{\Delta 54}$-His$_6$ (with and without the His$_6$ tag, **Figure 3.18 B** and **C**). However, in *T. brucei* lysates, the antisera cross-reacted with a non-specific band (~100 kDa) just above the highly abundant VSG (visible by SDS-PAGE, lane 7) and no band was detectable in the expected range for *Tb*NTR of 25 – 37 kDa. Hence, immunoprecipitation of endogenous *Tb*NTR with the available antiserum was not possible.

3.6.2. Pull-down of *Tb*NTR-*myc* from *T. brucei* lysates

An alternative approach for isolation of *Tb*NTR involved expression of the protein with a C-terminal tag in *T. brucei*, so that the tag would not interfere with mitochondrial processing at the N-terminus. It was expected that a subsequent pull-down with an appropriate antibody should allow identification of the mature protein start site. Based on a similar experiment, aimed at isolation of *Tb*GPI12*myc* (Güther *et al*., 2006), a *c-myc*-tagged tetracycline-inducible copy of *TbNTR* (^{Ti}*TbNTR-myc*) was introduced into wild-type BSF *T. brucei* under phleomycin selection. The resulting transgenic cell line was cloned and protein expression was induced with tetracycline in randomly selected

clones. In order to verify whether ^{Ti}*Tb*NTR-*myc* was expressed in an enzymatically active form, the EC_{50} value for nifurtimox was determined against the tetracycline-induced clones and compared to the EC_{50} against wild-type *T. brucei*. This step was necessary because the endogenous nitroreductase activity in *T. brucei* was below the limit of detection of the available spectrophotometric assay (unpublished observations). The ^{Ti}*Tb*NTR-*myc*-expressing clone which displayed the highest shift in EC_{50} (clone c2, **Figure 3.19 A**) was found to be 3-fold more sensitive to nifurtimox, compared to wild-type *T. brucei*. This shift in sensitivity was less that the approximately 10-fold shift in EC_{50} determined against BSF *T. brucei* expressing either a similarly tagged *Tb*NTR (Wilkinson *et al*., 2008) or a non-tagged ^{Ti}*Tb*NTR from an ectopic gene copy (see Chapter 4). Nonetheless, the result was sufficient to conclude that at least part of the tagged ^{Ti}*Tb*NTR-*myc* was processed in a manner similar to native *Tb*NTR and was able to cause a detectable change in nitroreductase activity.

To test the ability of the commercial anti-*myc* antibody to recognise ^{Ti}*Tb*NTR-*myc* in *T. brucei* lysates, a Western blot analysis was conducted (**Figure 3.19 B**). As expected, a distinct protein band migrating below the 37 kDa marker* was detected in the induced ^{Ti}*Tb*NTR-*myc*-expressing clone c2, but not in wild-type *T. brucei*. Although an additional, non-specific 50-kDa band was recognised in both transgenic and wild-type cells, the difference in size was deemed sufficient to discriminate between the two bands, should the 50-kDa protein co-precipitate with the anti-*myc* mAb. However, due to a number of technical difficulties, subsequent immunoprecipitation experiments carried out with the same anti-*myc* mAb failed to pull-down recombinant ^{Ti}*Tb*NTR-*myc*.

In summary, this chapter describes some of the numerous attempts that were made in an effort to obtain pure enzymatically active recombinant *Tb*NTR. These attempts included expression of full-length and various N-terminally truncated forms of

* The molecular weight of full-length, unprocessed ^{Ti}*Tb*NTR-*myc* is 37.0 kDa.

*Tb*NTR, with or without affinity and solubilisation tags at either the N- or the C-terminus, in several *E. coli* expression strains under a range of expression and lysis conditions, and even in cell-free lysates. Despite these endeavours, no pure, soluble, enzymatically active *Tb*NTR could be obtained, most probably due to the membrane association of the recombinant protein. Although further work, such as identification of the start site of mature *Tb*NTR, might reveal a solution to this problem, subsequent efforts were concentrated on studies of nitroaromatic drug resistance in *T. brucei*.

Chapter 4 - Studies in bloodstream form *T. brucei*

4.1. Functional studies of *Tb*NTR in bloodstream form *T. brucei*

4.1.1. Overexpression of *Tb*NTR in *T. brucei*

In the previous chapter, the nitroreductase activity of recombinant *Tb*NTR was demonstrated in clarified bacterial lysates. However, partly due to its membrane association, pure enzymatically active recombinant *Tb*NTR could not be obtained, although an inactive form of the recombinant protein was eventually purified. Furthermore, purification of recombinant His-tagged *Tb*NTR$_{\Delta 83}$, reported by Wilkinson and colleagues (Hall *et al*., 2010; Wilkinson *et al*., 2008), could not be reproduced for various reasons (see Chapter 5), potentially including differences in the amino acid sequence. Therefore, to further validate that the sequence variant of *Tb*NTR used in our studies (Thr57/Asp69/Asn261) can function as a nitroreductase, this variant was overexpressed in BSF *T. brucei* and the drug sensitivity of the resulting transgenic line was compared to that of wild-type and of *TbNTR* single knock-out *T. brucei*.

To create an NTR-overexpressing cell line of *T. brucei*, a tetracycline-inducible ectopic copy of the gene (^{Ti}*TbNTR*) was introduced into wild-type BSF cells under phleomycin selection (**Figure 4.1 A**). The transgenic line was cloned, and expression of ^{Ti}*Tb*NTR was induced in three of the clones by addition of tetracycline to the culture media for at least one week. Induced clones were found to be approximately 10-times more sensitive to nifurtimox than wild-type cells, with EC_{50} values in the range of 0.17 – 0.21 μM (**Figure 4.1 B**), compared to 1.8 μM against wild-type *T. brucei*. Due to lack of *Tb*NTR-specific antibodies (section 3.6.1) or other methods for detection of NTR in *T. brucei* lysates, such as a sufficiently sensitive enzyme assay, the increased sensitivity to nifurtimox could not be correlated directly to increased NTR levels. Nonetheless, the substantial shift in the EC_{50} value for nifurtimox against induced ^{Ti}*Tb*NTR-expressing cells indicated that this particular sequence variant (Thr57/Asp69/Asn261) is functionally active in *T. brucei* in terms of nifurtimox activation.

4.1.2. *TbNTR* single knock-out cell lines

Prior to generation of *TbNTR* SKO lines, it was demonstrated that the gene is present as a single copy per haploid genome in *T. brucei* S427 (section 3.1.2), as annotated for the genome reference strain of *T. brucei* TREU927. Following from these results, one of the two alleles of the *TbNTR* gene was replaced by homologous recombination with a selectable marker gene (either *HYG* or *PAC*; **Figure 4.2 A**), resulting in the transgenic *T. brucei* lines SKO^{HYG} and SKO^{PAC}. To verify that the marker genes were integrated at the *NTR* locus, gDNA extracted from wild-type cells and from SKO^{HYG} and SKO^{PAC} clones was analysed by PCR. A single product at the expected size of 1.2 kb was amplified from wild-type gDNA using primers 5′UTR_-107_F and 3′UTR_+152_R (**Table 2.1**), which align to sequences within the 5′- and 3′-UTRs of the replacement cassette (**Figure 4.2 B**). An additional product at either 1.3 kb or 0.87 kb was amplified with the same primer pair from the gDNA of SKO^{HYG} clone 2 and SKO^{PAC} clone 1 respectively, which confirmed the presence of the selectable marker genes in the SKO clones. Similar results were observed with primers 5′UTR_-595_F and 3′UTR_+600_R, aligned to sequences outside the recombination region. PCR with this primer pair resulted in a single 2.16 kb band when wild-type gDNA was used as a template, whereas using gDNA template from SKO^{HYG} clone 2 and SKO^{PAC} clone 1 resulted in an additional band at 2.2 kb and at 1.8 kb, respectively. Collectively, these findings confirmed that one allele of *TbNTR* had been knocked-out and replaced by the selectable marker gene in both SKO clones.

To investigate how reduction of the *NTR* gene copy number affects the ability of BSF *T. brucei* to process nifurtimox and other nitroaromatic compounds, the drug sensitivity of the cloned SKO lines was examined. In two independent experiments, SKO^{HYG} clone 2 was found to be 1.7- and 1.6-fold less sensitive to the drug than wild-type *T. brucei*, whereas in the same experiments SKO^{PAC} clone 1 was less sensitive than

the wild-type cells by 1.9- and 2.3-fold, respectively (**Figure 4.2 C**). These results are comparable with published data for other *NTR* SKO lines of *T. brucei* (Wilkinson *et al*., 2008), where up to 3-fold reduction in sensitivity was reported. Having established that the sensitivity to nifurtimox is dependent on the level of *NTR* copy number in *T. brucei*, future work will focus on examining how the sensitivity to other nitroaromatic drugs, such as the clinical candidate fexinidazole, is affected under similar circumstances.

4.2. Resistance to nitroaromatic drugs in *T. brucei* *

4.2.1. Generation of nifurtimox-resistant *T. brucei* cell lines

In order to investigate the potential of BSF *T. brucei* to acquire resistance to nifurtimox, cells were cultured under the continuous selective pressure of increasing drug concentrations (**Figure 4.3 A**). Initially, trypanosomes were exposed to a sublethal concentration of 1.5 μM nifurtimox, which reduces cell growth in wild-type *T. brucei* by less than 25%. Once the cells were able to grow at normal rates in the presence of a low concentration of nifurtimox, they were introduced to a higher, usually double drug concentration. After a total of 140 days in the presence of drug, the cells were able to grow routinely in 50 μM nifurtimox, which is more than 3-times the EC_{99} value against wild-type *T. brucei*. At this stage, the resulting nifurtimox-resistant line, designated NfxR, had a doubling time in the absence of drug of 8 h, which was similar to the 7.3-h doubling time of wild-type *T. brucei* (**Figure 4.3 B**). By contrast, in the presence of 50 μM, NfxR had an increased doubling time of 12.8 h, whereas the parental wild-type cells died within 3 – 4 days of exposure to the same drug concentration. These results confirmed that NfxR were indeed resistant to nifurtimox and that generating such resistance did not affect the cells ability to grow in culture.

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* Part of this section has been published in Sokolova *et al*., 2010 (**Appendix**).

To determine the exact extent of nifurtimox resistance of NfxR, several clones were derived from this cell line in the absence of drug immediately following the 140-day selection process. The two most resistant clones, NfxR1 and NfxR2, had EC_{50} values for nifurtimox of 20.1 ± 0.9 μM and 20.3 ± 1.3 μM, respectively, which is 8-fold higher than the EC_{50} against wild-type *T. brucei* (2.4 ± 0.1 μM; **Figure 4.4**). Although NfxR1 and NfxR2 were cloned and routinely cultured in the absence of the selective drug, they retained their resistant phenotype for over 60 days (30 passages) in culture.

The resistance of these clones appeared to be specific for nifurtimox, as their sensitivity to the non-nitroaromatic drugs pentamidine and eflornithine was comparable (within 3-fold) with that of the wild-type parental strain (**Figure 4.4** and **Table 4.1**). Initially, NfxR1 and NfxR2 appeared to be more sensitive than wild-type cells to eflornithine, with EC_{50} values of 11.9 ± 0.7 μM and 11.5 ± 0.6 μM versus 16.4 ± 0.6 μM, respectively. The difference in these EC_{50} values between the nifurtimox-resistant and -sensitive cells was found to be statistically significant ($p < 0.01$, Student's *t*-test) only when the weighted mean EC_{50} values were compared. However, when the range of individual EC_{50} determinations was taken into account (10.2 – 16.8 μM against resistant and 11.0 – 19.8 μM against sensitive cells), no such significance was found ($p > 0.17$). In the case of pentamidine, the weighted mean EC_{50} values were found to be statistically significantly different ($p < 0.001$) not only between nifurtimox-resistant and -sensitive cells, but also between NfxR1 and NfxR2. However, similar to eflornithine, the individual EC_{50} values for pentamidine, which were in the range of 1.3 – 5.0 nM against NfxR clones and 0.7 – 6.1 nM against wild-type cells, showed no statistically significant difference ($p > 0.7$).

4.2.2. Cross-resistance to nitroaromatic compounds *in vitro*

To determine whether generation of nifurtimox resistance in *T. brucei* can confer cross-resistance to other nitroaromatic drugs, the sensitivity of Nfx1 and NfxR2 to a number of compounds was examined (**Table 4.1**). Both clones were found to be cross-resistant to nitrofurazone, with a resistance factor of 12, similar to that for the related drug nifurtimox. Nitrofurazone is overall more potent than nifurtimox against *T. brucei*, but it is also highly toxic in humans, which has led to the discontinuation of its use as a treatment for second-stage sleeping sickness soon after it was introduced (Williamson, 1970). More importantly, however, cross-resistance was observed to the current clinical candidate fexinidazole, which measured the highest resistance factor of the tested compounds (27- and 29-fold for NfxR1 and NfxR2, respectively). Furthermore, the NfxR clones were highly resistant (up to 15-fold) to the two proposed metabolites of fexinidazole, the sulfoxide and sulfone (Winkelmann and Raether, 1977; Winkelmann and Raether, 1978). In addition, NfxR1 and NfxR2 were cross-resistant (by a factor of 5) to benznidazole, which has a relatively low potency against wild-type *T. brucei* (EC_{50} = 30.0 ± 0.2 μM) and is currently only used against acute or early-chronic infections with *T. cruzi*. By contrast, NfxR1 and NfxR2 were not resistant to the anti-tubercular nitrodrug PA-824 (Singh *et al*., 2008), with EC_{50} values in the range of 30 – 40 μM against both NfxR and wild-type *T. brucei*. Another antibacterial and antiprotozoal drug, metronidazole, which is primarily used against microaerophilic organisms (Löfmark *et al*., 2010), was ineffective against both wild-type and NfxR cells even at highest test concentration of 500 μM. The final test compound was the anticancer pro-drug CB1954, which was first identified as an antitrypanosomal agent during a phenotypic screen in our laboratory (Jones *et al*., 2010b). Furthermore, CB1954 is a substrate for the bacterial nitroreductases which are known to activate nitrofurazone (Anlezark *et al*., 1992). Both NfxR1 and NfxR2 were found to be resistant to CB1954

by factors of 15- and 19-fold respectively, similar to the resistance factors obtained with nitrofurazone and the fexinidazole metabolites against these clones. In summary, these results demonstrate that generation of nifurtimox resistance in BSF *T. brucei* has led to high levels of cross-resistance to several nitroaromatic compounds, which belong to three related chemical classes and are thought to share a similar mode of action and mode of activation with nifurtimox.

4.2.3. Cross-resistance to nitroaromatic compounds *in vivo*

The biological relevance of nifurtimox resistance generated *in vitro* in NfxR cells was tested in several mouse-model studies. In a pilot experiment, performed by Dr Susan Wyllie, University of Dundee, NMRI mice were infected with wild-type, NfxR1 or NfxR2 *T. brucei* (10^4 cells per mouse, 5 mice per group) and monitored daily for signs of infection. Mice infected with wild-type cells succumbed to infection within 5 to 6 days, with a mean survival time of 5.2 ± 0.4 days. Mice infected with NfxR1 and NfxR2 had almost identical survival times (5.2 ± 0.4 and 5.4 ± 0.9 days, respectively), which indicated that NfxR cells were able to establish fatal levels of parasitaemia at the same rate as wild-type cells. Therefore, the virulence of these nifurtimox-resistant cells was not compromised in the process of generating such resistance.

A second study, also carried out by Dr Susan Wyllie, aimed to determine whether NfxR cells were resistant to nifurtimox *in vivo*. Mice (n = 5 per group), infected with NfxR1 or wild-type *T. brucei* (10^4 cells per mouse), were either left untreated as controls or given a 4-day course of 100 mg kg^{-1} day^{-1} (i.p.) nifurtimox, starting 24 h post infection (**Figure 4.5 A**). This regimen was based on the 99% effective dose of nifurtimox ($ED_{99} \approx 128$ mg kg^{-1} day^{-1}), calculated using equation (7) and published data on ED_{50} (36.7 mg kg^{-1} day^{-1}) and Hill slope ($m = 3.7$) values for nifurtimox (Fairlamb *et al*., 1992). As expected from the pilot study, the control groups, which did not receive

treatment, succumbed to infection by either wild-type or NfxR1 cells within 5 to 7 days. Furthermore, one mouse from each group that received nifurtimox had to be sacrificed as it experienced severe adverse side effects caused by the drug, which suggested that 100 mg kg^{-1} day^{-1} was at the limit of or exceeding the maximum tolerated dose for nifurtimox in these animals. From the mice which survived treatment with nifurtimox, those infected with wild-type *T. brucei* were cured by this treatment and shown to be free from parasitaemia for the duration of the experiment (a total of 30 days). However, mice infected with NfxR1 and treated with nifurtimox succumbed to infection within the same time period as mice that did not receive the drug. Collectively, these results indicate that, even at the highest tolerated dose, nifurtimox has no effect on the growth of NfxR1 cells, which remained resistant to this drug both *in vitro* and *in vivo*.

To determine whether NfxR1 were also cross-resistant to fexinidazole *in vivo*, a similar study was performed by Dr Kevin Read and the DMPK group at the University of Dundee. In this case, mice infected with either NfxR1 or wild-type *T. brucei* received 4 doses of oral fexinidazole ranging from 25 to 200 mg kg^{-1} day^{-1} and starting on day 1 post infection (**Figure 4.5 B**). Data for the control groups, which did not receive treatment, is presented as a 0 mg^{-1} kg^{-1} dose. Unlike nifurtimox, fexinidazole had no adverse effects in these mice even at the highest administered dose, which suggested that fexinidazole is better tolerated than nifurtimox. Fexinidazole also appeared to be more effective, because it had a lower ED_{50} value (21 mg^{-1} kg^{-1} day^{-1}; m = 7.9) against wild-type cells and an ED_{99} of 37.6 mg^{-1} kg^{-1} day^{-1}, which would only result in a ~50% cure rate if applied for nifurtimox. Nonetheless, similar to nifurtimox, fexinidazole was not effective against NfxR1 infection at its ED_{99} dose. The ED_{50} of fexinidazole against NfxR1 cells in mice was estimated to be 171 mg^{-1} kg^{-1} day^{-1}, 8.5-fold lower than that against the parental wild-type cells. This estimate was based on the survival of 3 out of 5 mice infected with NfxR and dosed with 200 mg kg^{-1} day^{-1} fexinidazole. However,

only two of those mice were cleared of parasitaemia as a result of the treatment, whereas the third mouse experienced several relapses between end of treatment (day 4) and day 18. Eventually, this mouse appeared to be able to overcome the infection by itself and survived until the termination of the experiment on day 60.

In conclusion, the above results confirm that generation of resistance to nifurtimox in BSF *T. brucei* can confer *in vivo* cross-resistance to the clinical candidate fexinidazole.

4.2.4. Generation of a fexinidazole-resistant *T. brucei* line

To investigate whether the relationship between nifurtimox and fexinidazole resistance is reciprocal, a fexinidazole-resistant *T. brucei* line was generated essentially as described for NfxR (section 4.2.1). Wild-type trypanosomes were initially exposed to a sublethal concentration of fexinidazole (1 μM), which was subsequently increased in a step-wise manner as the cells adapted their growth (**Figure 4.6 A**). Similar to NfxR, the resulting fexinidazole-resistant line (FxR) was adapted to growth in 50 μM drug after a total of 137 days in culture, with the notable exception that FxR was able to survive an increase in drug concentration from 25 μM to 50 μM, whereas NfxR required an intermediate concentration step (**Figure 4.3 A** and **4.6 A**). Clones FxR1 and FxR2, derived from this line at the end of the selection period, were almost 10-fold less sensitive to fexinidazole compared to the parental wild-type cells (**Figure 4.6 B**). The respective weighted mean EC_{50} (and Hill slope) values against these clones were 16.9 ± 0.8 μM (m = 1.5) and 16.0 ± 1.2 μM (m = 1.2), whereas in the same experiment (four independent determinations), the weighted mean EC_{50} against wild-type *T. brucei* was 1.7 ± 0.1 μM (m = 1.6; **Table 4.2**). Similar data were obtained for nifurtimox in three independent determinations, where the weighted mean EC_{50} values against FxR1 and FxR2 (19.0 ± 0.8 μM, m = 2.8 and 18.6 ± 0.7 μM, m = 2.3, respectively) were 9- to 10-

fold higher than the corresponding EC_{50} value against the parental cells (2.0 ± 0.1 μM, m = 2.8).

These results emphasise the fact that in both wild-type and nitrodrug-resistant cells the Hill slope for nifurtimox is consistently steeper than that for fexinidazole. Consequently, the relationship between the EC_{50} values for these two compounds against a given cell line will not be the same as their corresponding EC_{99} values. For example, against wild-type *T. brucei* fexinidazole has a 2.4-fold lower EC_{50} than nifurtimox, which would suggest that fexinidazole is the more potent compound *in vitro*. However, the actual concentration required for 99% growth inhibition of wild-type cells is very similar for both nifurtimox and fexinidazole (15.2 μM versus 17.8 μM, respectively). By contrast, in all nitrodrug-resistant clones, nifurtimox and fexinidazole have similar EC_{50} values, but in each case, the EC_{99} for fexinidazole is higher than that for nifurtimox. Therefore, despite almost identical EC_{50} data for the two compounds, nitrodrug-resistant cells can survive much higher levels of fexinidazole than of nifurtimox.

The second implication of the above study comes from the different resistance factors determined for NfxR and FxR clones. It appears that when resistance was generated to nifurtimox, the resulting NfxR cells were more resistant to fexinidazole than they were to nifurtimox, when judged by either their RF_{50} or their RF_{99} resistance factors. By contrast, when the selection drug was fexinidazole, the resulting FxR cells developed almost equal levels of cross-resistance to both drugs. This observation indicates that FxR cells may have a different mode of resistance to nitroaromatic drugs, compared to NfxR cells.

4.2.5. Pharmacokinetic studies of fexinidazole

When fexinidazole was initially considered as a therapeutic option for African sleeping sickness, it was suggested (Winkelmann and Raether, 1978) that in mammals this compound is most probably metabolised to sulfoxide and sulfone derivatives via oxidation of the sulphur atom (**Figure 4.7 A**). To test this hypothesis, Dr Kevin Read and the DMPK team at the University of Dundee analysed blood samples from mice dosed with a single oral dose of 200 mg kg^{-1} fexinidazole. Data revealed that fexinidazole reached a total concentration peak at 960 ng ml^{-1} (~3.4 μM) in the first half an hour after dosing (**Figure 4.7 B**). Soon after fexinidazole levels started to decline (1 – 2 h after dosing), fexinidazole sulfoxide was found to peak at the much higher total concentration of 40,000 ng ml^{-1} (135 μM). The subsequent steady decline of the levels of fexinidazole and its sulfoxide derivative was coincident with a prolonged and sustained increase in the level of the second metabolite, fexinidazole sulfone, which reached 55,000 ng ml^{-1} (176 μM) when last measured 8 h after dosing. Based on these results, it was evident that the maximum peak concentration of fexinidazole, following a single oral dose, is far below its EC_{99} value against wild-type *T. brucei* (4,900 ng ml^{-1} or 17.8 μM) determined from previous *in vitro* studies (**Table 4.2**). By contrast, both fexinidazole sulfone and sulfoxide were present at concentrations much higher than their corresponding EC_{99} values (7,300 ng ml^{-1} and 3,400 ng ml^{-1}, or 25 μM and 11 μM, respectively) for a period of over 6 h. These observations hold true even if only the free, unbound fraction (f_u) of the drug and its metabolites in plasma is taken in account. A separate study by Dr Kevin Read and the DMPK group established that f_u of fexinidazole is 0.147, whereas fexinidazole sulfoxide and sulfone have much higher values for f_u (0.858 and 0.728, respectively; Sokolova *et al*., 2010). Collectively, these findings confirm that fexinidazole is unlikely to act as the bioactive agent against *T.*

brucei. Instead, most of the trypanocidal activity *in vivo* is probably exerted by its sulfoxide and sulfone metabolites.

4.3. Role of *Tb*NTR in resistance to nitroaromatic drugs in *T. brucei*

4.3.1. Southern analysis of gDNA from nitrodrug-resistant and -sensitive lines

A recent study on laboratory-generated nifurtimox-resistant *T. cruzi* has implicated loss of the nitroreductase gene, *TcNTR* (Tc00.1047053510611.60), as a potential mechanism of resistance to nitroaromatic drugs (Wilkinson *et al*., 2008). To investigate whether the *T. brucei* homologue of *TcNTR* has been affected in our NfxR and FxR cell lines in the process of generating such resistance, gDNA extracted from these cells, as well as from wild-type *T. brucei*, was analysed by Southern hybridisation using two reference genes (**Figure 4.8 A**). These genes were selected so that one of them (*TbDHFR-TS*) was positioned on the same chromosome as *TbNTR* and the other (*TbTRYS*) was on a different chromosome. Furthermore, both *TbDHFR-TS* and *TbTryS* are single copy genes per haploid genome (Gamarro *et al*., 1995a; Gamarro *et al*., 1995b; Oza *et al*., 2003), and most importantly, they are thought to be unrelated to the mode of action and mode of activation of nitroaromatic pro-drugs.

Prior to the hybridisation analysis, agarose gel electrophoresis of the EcoRV-digested samples revealed that the gDNA of NfxR1 had a different restriction pattern of fragments below 2 kb (**Figure 4.8 B**), compared to wild-type gDNA. This indicated that NfxR1 cells had undergone some form of genetic modification, although at this stage no distinction could be made between insertions/deletions of DNA fragments and mutations at certain EcoRV recognition sites.

Following hybridisation with the labelled ORF of *TbNTR*, a band at the expected size of 6.8 kb was observed in all samples (**Figure 4.8 C**), which confirmed that at least one allele of *NTR* was present in both NfxR and FxR cell lines. To determine whether

changes in the number of *TbNTR* alleles have occurred in these cell lines, the intensity ratio of the band corresponding to *TbNTR* and the bands corresponding to the two reference genes was determined in each gDNA sample (**Figure 4.8 C**). Based on comparison of these ratios between the resistant lines and wild-type *T. brucei* (**Figure 4.8 C**), it was evident that only FxR2 cells had the same proportion of all three gene sequences as in wild-type cells, which would suggest that no change in the *NTR* copy number had occurred in these cells. The other FxR clone (FxR1) was found to have altered band intensity ratios of *DHFR-TS* to both *NTR* and *TryS*, whilst the ratio *NTR:TryS* was similar to that in wild-type cells. In theory, this would suggest that FxR1 cells had lost an allele of *DHFR-TS*, or lost part of chromosome 7 containing *DHFR-TS* but not *NTR*. An alternative, but highly unlikely explanation would be that both *NTR* and *TryS* copy numbers had been increased by an equal amount and no change had occurred at the *DHFR-TS* locus. In each case, this result is unexpected and highlights potential technical issues with the experiment, most probably at the stage of determining the individual band intensities. Much more logical data were obtained with the two NfxR clones, where the band intensity ratio of *NTR* to each of the two reference genes was reduced by approximately 1.5-fold, relative to the corresponding ratios in wild-type *T. brucei*. Similar to the above results, this was an indication that one allele of *NTR* was lost in both NfxR1 and NfxR2 cells. However, in this case the data was consistent with the expected role of NTR in activation of nitroaromatic pro-drugs. Although these results require confirmation, they indicate that the four nitrodrug-resistant clones are genetically different from one another. Interestingly, the main difference between the NfxR and FxR clones appears to be in the partial loss of *TbNTR* in the former.

4.3.2. Sequence analysis of gDNA from nitrodrug-resistant and -sensitive lines

To determine whether any alterations had occurred in the *TbNTR* sequence of nitrodrug-resistant cells, the ORF of *TbNTR*, flanked by 100 bp at the 5′ terminus and 150 bp at the 3′ terminus, was amplified by PCR from gDNA and analysed by sequencing. As previously reported (section 3.1.1), *TbNTR* from wild-type *T. brucei* was found to be polymorphic at four nucleotide positions, which presented as double peaks of equal signal intensity on the sequencing electropherogram (**Figure 4.9**). By contrast, in NfxR1 and NfxR2 cells, which had lost one allele of *TbNTR* (section 4.3.1), only one sequence variant of the gene was present (Thr57/Asp69/Asn261), and consequently only a single nucleotide peak was observed at each SNP position. Furthermore, in both NfxR clones the remaining allele of *TbNTR* was found to contain a unique point mutation (G316A; black asterisks on **Figure 4.9**), which was not present in any of the FxR clones, and which results in the amino acid substitution Val106 to Ile. This was the only mutation found in *TbNTR* from nitrodrug-resistant cells, because the sequence of *NTR* from the FxR clones was identical to that from the wild-type parental cells. Since the FxR clones had more than one allele of *TbNTR*, they also displayed polymorphism at all four SNP sites identified in wild-type cells. From these data, it could be concluded that *TbNTR* was not affected in the process of generating *in vitro* resistance to fexinidazole in BSF *T. brucei*, whereas using a similar process to generate nifurtimox resistance in these cells resulted in loss of one *TbNTR* allele and a substitution mutation in the remaining allele.

4.3.3. Analysis of *Tb*NTR$_{Val106Ile}$

The gene encoding *Tb*NTR in all NfxR clones was found to contain a point mutation which affects its amino acid sequence. Ideally, to determine whether this particular amino acid change (Val106Ile) affects the nitroreductase activity of the resulting mutant

form (*Tb*NTR$_{Val106Ile}$), purified recombinant protein would be required. However, numerous attempts to obtain recombinant *Tb*NTR have failed to produce pure and at the same time enzymatically active protein (see Chapter 3). Therefore, to circumvent the need for protein purification, the ability of recombinant *Tb*NTR$_{Val106Ile}$ to reduce nitroaromatic compounds was examined in whole cell *E. coli* using a specifically adapted disc diffusion test. For this purpose, *Tb*NTR$_{Val106Ile}$ was expressed as a hexahistidne-tagged, truncated form (*Tb*NTR$_{Val106Ile_\Delta54}$-His$_6$), corresponding to the truncated form of non-mutant *Tb*NTR that was previously shown to have a detectable nitroreductase activity in *E. coli* lysates (*Tb*NTR$_{\Delta54}$-His$_6$; see sections 3.4.4 and 3.4.5). The sensitivity of the resulting *E. coli* strains, expressing either *Tb*NTR$_{\Delta54}$-His$_6$ or *Tb*NTR$_{Val106Ile_\Delta54}$-His$_6$, was determined for nitrofurazone, at concentrations of 0.1, 1 and 10 mM (5 µl per disc, **Figure 4.10 A**). The disc diffusion test was also performed with nifurtimox, although this compound had to be applied at 10-fold higher concentrations to produce similar-sized zones of inhibition (**Table 4.3**). In each case, there was a clear zone with no visible growth around the disc with the highest test concentrations, whereas the intermediate concentrations only reduced the colony size and number around the disc (data in brackets in **Table 4.3**), and the lowest test concentrations failed to produce any visible zone of inhibition. By contrast, fexinidazole failed to produce clear zones of inhibition in any of the *E. coli* strains even at 100 mM (not shown), and is not discussed further. The drug carrier DMSO did not produce any visible reduction in growth, which suggested that the observed zones of reduced or absent growth were the result of inhibition by the drug itself. As predicted, in positive control cells expressing *Ec*NfsB, the zones of inhibition for nitrofurazone were broader, indicating higher drug sensitivity, compared to cells with background nitroreductase activity alone (transformed with an empty vector). A comparable increase in the zone of inhibition was also observed in cells expressing either wild-type or mutant *Tb*NTR$_{\Delta54}$-

His_6, thereby confirming that both of these proteins were capable of reducing and activating nitrofurazone. Interestingly, only cells expressing *Tb*$NTR_{\Delta 54}$-His_6 or *Tb*$NTR_{Val106Ile\_\Delta 54}$-$His_6$, but not *Ec*NfsB, displayed a marginal increase in their zone of inhibition for nifurtimox, visible at the highest test concentration (100 mM). A possible explanation is that nifurtimox is a good substrate for *Tb*NTR and a poor one for *Ec*NfsB, although this would require further investigation to confirm. In any case, the above experiments demonstrate that mutant *Tb*$NTR_{Val106Ile}$ is enzymatically active and can reduce nitroaromatic drugs, at least when expressed in *E. coli*.

To verify that *Tb*$NTR_{Val106Ile}$ is also enzymatically active in *T. brucei*, a tetracycline-inducible ectopic copy of the mutant gene was introduced into wild-type cells under phleomycin selection as previously described (section 4.1.1). All tetracycline-induced clones, derived from the resulting *Tb*$NTR_{Val106Ile}$-overexpressing *T. brucei* cell line, were found to be 3- to 5-fold more sensitive to nifurtimox than the parental wild-type cells (see example in **Figure 4.10 B**). The mean EC_{50} (and Hill slope) values determined for all clones on two separate occasions were 0.36 ± 0.06 µM (m = 2.6) and 0.46 ± 0.08 µM (m = 2.1), with corresponding EC_{50} values of 1.3 ± 0.1 µM (m = 1.8) and 1.9 ± 0.1 µM (m = 2.2) against wild-type cells. The consistent shift in nifurtimox sensitivity confirmed that mutant *Tb*$NTR_{Val106Ile}$ was capable of activating nifurtimox in BSF *T. brucei*. Therefore, the synonymous mutation (Val106Ile) present in NfxR1 and NfxR2 clones cannot account for nitrodrug resistance in these cells.

In summary, this chapter demonstrated that resistance to either nifurtimox or fexinidazole could be generated easily in BSF *T. brucei*, and that the resulting resistant cell lines were cross-resistant to a range of nitroaromatic pro-drugs. Such reciprocal cross-resistance indicated the presence of common resistance mechanisms shared by both cell lines. However, there were also clear differences between NfxR and FxR *T. brucei*, as demonstrated by variation in the resistance factors determined for each cell

line and by the genetic differences found at the *NTR* locus. Unravelling further mechanisms of resistance would be a priority for future investigations, which may not only contribute to better understanding of the biology of trypanosomes, but may also help to prevent such resistance from occurring in the field.

Chapter 5 -

Discussion

5.1. Key findings

The lack of better and safer chemotherapeutic options for African sleeping sickness has led to the re-introduction of nitroaromatic compounds, such as nifurtimox (in combination with eflornithine, NECT) and fexinidazole (undergoing clinical trials), for use against the second stage of the disease (Torreele *et al*., 2010). However, relatively little is known about the implications of widespread use of these drugs against HAT, especially about the resistance potential of this class of compounds in the African trypanosome. Furthermore, the mode of action of nitroaromatic pro-drugs against trypanosomes remains poorly understood, with studies almost exclusively carried out in the South American *T. cruzi*. To address some of these unknowns, I have undertaken the current study, in which I have demonstrated that:

- It is relatively easy to generate *in vitro* resistance nifurtimox in BSF *T. brucei*, without loss of virulence
- The resulting NfxR cells are cross-resistant (to a variable degree) to a number of nitroaromatic compounds *in vitro*, as well as to nifurtimox and fexinidazole *in vivo*
- Conversely, it is relatively easy to generate resistance to fexinidazole, and the resulting FxR cells are cross-resistant to both fexinidazole and nifurtimox *in vitro*
- Cross-resistance to nifurtimox and fexinidazole indicates that the two pro-drugs share some common mechanisms of action, activation and resistance
- Nonetheless, there appear to be a subtle difference in these mechanisms, depending on which drug was used to generate resistance, nifurtimox or fexinidazole
- Loss of a *TbNTR* gene copy is only partially responsible for resistance to nifurtimox (but not to fexinidazole), and point mutations in this gene seem unlikely to play any role in nitrodrug resistance
- Purification of enzymatically-active *Tb*NTR could not be achieved, as recombinant *Tb*NTR was found to be insoluble and membrane-associated.

5.2. Mode of action of nitroaromatic pro-drugs in *T. brucei*

5.2.1. Mechanisms of action of nitroaromatic pro-drugs in trypanosomes

Nitroheterocyclic drugs have been used against South American trypanosomiasis since the 1970s (Fries and Fairlamb, 2003). Many attempts have also been made since the 1950s to introduce these drugs for the treatment of HAT (Pépin and Milord, 1994; Williamson, 1962), culminating in the replacement of eflornithine with NECT for use against the second disease stage (Yun *et al*., 2010). Nonetheless, the precise mode of action of nitroaromatic drugs in trypanosomes is still unclear. Pioneering work by Docampo and co-workers (Docampo *et al*., 1981; Docampo and Stoppani, 1979) has suggested that nitrofurans, in particular nifurtimox, act via the formation of nitro anion radicals, which undergo futile redox cycling to generate reactive oxygen species in *T. cruzi*. However, this hypothesis has been challenged in a recent study (Boiani *et al*., 2010), which argued that growth-inhibitory concentrations of nifurtimox did not induce the formation of reactive oxygen species. The mode of action of nitroimidazoles, such as benznidazole and megazol, in trypanosomes is also uncertain, but appears to be different from that of nitrofurans and to involve damage to macromolecules and depletion of intracellular thiols (Diaz-de-Toranzo *et al*., 1988; Enanga *et al*., 2003; Maya *et al*., 2003).

Despite the vast uncertainties regarding the mode of action of nitroaromatic pro-drugs in trypanosomes, it is universally accepted that the first step in the process is drug activation by enzyme-mediated reduction of the nitro group. Several different enzymes, which catalyse electron transfer reactions, have been proposed to perform this initial step. Both recombinant TryR (from *T. cruzi* and the insect-infective *Crithidia fasciculata*) and recombinant lipoamide dehydrogenase (from *T. cruzi*) have been shown to conduct one-electron reduction of nifurtimox and other nitroaromatic derivatives (Blumenstiel *et al*., 1999; Henderson *et al*., 1988). However, activation of nitroaromatic pro-drugs by

lipoamide dehydrogenase has not been demonstrated in intact cells, whereas overexpression of TryR was found to have no effect on nifurtimox sensitivity in *T. cruzi*, suggesting that TryR did not play a significant role in the bioactivation of nitrodrugs (Hall *et al*., 2011; Kelly *et al*., 1993). Another flavoprotein, *Tc*OYE, was found to catalyse two-electron reduction of nifurtimox only under anaerobic conditions, both as a purified enzyme and in *T. cruzi* cell lysates (Kubata *et al*., 2002). Interestingly, *Tc*OYE was not able to catalyse reduction of benznidazole, yet the gene and protein levels of OYE were reduced in two benznidazole-resistant strains of *T. cruzi* (Andrade *et al*., 2008; Murta *et al*., 2006). Even though this enzyme may be required for bioactivation of nitrofurans in *T. cruzi* (under anaerobic conditions), there are no homologues of *TcOYE* in the genome of *T. brucei* (http://www.genedb.org). Finally, the NADPH-cytochrome P450 reductase *Tc*CPR-B was found to act not as a bioactivator but as a detoxifying enzyme, conferring protection from benznidazole, and to a lesser extent from nifurtimox (Portal *et al*., 2008). By contrast, neither prostaglandin $F_{2\alpha}$ synthase (*Tb*PGS; non-homologous to OYE) nor two homologues of *Tc*CPR-B (*Tb*CPR2 and *Tb*CPR3) were found to have any effect on the sensitivity of *T. brucei* to nifurtimox (Hall *et al*., 2011). Thus, the only remaining candidate that can realistically act as a bioactivator of nitroaromatic pro-drugs in *T. brucei* is the newly uncovered bacterial-like nitroreductase *Tb*NTR (Tb927.7.7230).

In this study, I have demonstrated the role of *Tb*NTR as a bioactivator by overexpression of the protein in BSF *T. brucei*, resulting in a ~10-fold increase in sensitivity to nifurtimox. I have also shown that deletion of a *Tb*NTR copy by gene knock-out results in parasites that are 1.6- to 2.3-fold less sensitive, as would be expected for loss of a major activating enzyme. These results are in good agreement with studies by Wilkinson and colleagues, which have shown up to 3-fold loss of sensitivity in *Tb*NTR SKO cell lines, and a 10-fold increase in sensitivity to nifurtimox

and other nitroaromatics in cells overexpressing C-terminally tagged *Tb*NTR (Wilkinson *et al*., 2008). Furthermore, generation of *in-vitro* resistance to nifurtimox has resulted in loss of an *NTR* gene copy in *T. brucei* (this study) and loss of a chromosome containing *NTR* in *T. cruzi* (Wilkinson *et al*., 2008). Moreover, depletion of *Tb*NTR by RNA interference has resulted in resistance to nifurtimox (Baker *et al*., 2010). In studies with recombinant protein, *Tc*NTR has been shown to reduce nifurtimox, nitrofurazone, nitrofurantoin, benznidazole and megazol, whereas *Tb*NTR has been shown to activate nifurtimox and CB1954 (Bot *et al*., 2010; Wilkinson *et al*., 2008). The final reduction products, generated through reduction by *Tb*NTR, were identified as the 2- and 4-hydroxylamino derivatives of CB1954 (Bot *et al*., 2010) and speculatively as the open nitrile chain of nifurtimox (Hall *et al*., 2011), although the formation of these products in *T. brucei* needs to be confirmed. These recent studies, combined with my current work, have greatly enhanced our understanding of the mechanism of nifurtimox activation in trypanosomes, and the role of *Tb*NTR therein. In the near future, it will be imperative to carry out similar investigations into the mechanisms of action and resistance of fexinidazole.

5.2.2. Studies with recombinant *Tb*NTR

Nitroaromatic pro-drugs have recently experienced a revival, not only for used against African sleeping sickness, but also as effective antimicrobial, antiviral and anticancer agents. This diversity in the clinical applications of nitro drugs stems from the fact that their only requirement for biological activity is a nitro group attached to an aromatic moiety, thus leaving plenty of scope for modification of the aromatic structure (benzene, furan, imidazole, etc.) and its substituents to alter drug specificity and toxicity (Grunberg and Titsworth, 1973). This chemical flexibility can hopefully be exploited for the development of second-generation nitro pro-drugs, with increased potency and

lower toxicity, for use against HAT. However, for this to be achieved, detailed knowledge of the mechanisms of activation and action of these compounds will be required. This was partly resolved with the help of the genome-sequencing project (Berriman *et al*., 2005), which led to the identification of the key activator of these nitro pro-drugs in trypanosomes – the bacterial-like enzyme NTR. What is lacking to date is a crystal structure of this enzyme to guide structure-based drug design. The limiting factor has proven to be the lack of high quantities of purified recombinant enzyme. In the course of the current study, Wilkinson and colleagues reported on the expression and purification of both *Tb*NTR and *Tc*NTR, with protein yields that might have been enough for preliminary kinetic analysis, but were insufficient for crystallographic studies (Hall *et al*., 2010; Wilkinson *et al*., 2008). In fact, using what little information was provided in the published protocol (Wilkinson *et al*., 2008), I was not able to reproduce the expression and purification (no protein was recovered) of the truncated form of *Tb*NTR* studied by these authors. By contrast, I have successfully obtained pure and enzymatically active recombinant *Ec*NfsB by following previously published methodology (Anlezark *et al*., 1992; Zenno *et al*., 1996). The reasons for the discrepancies between my work and that of Wilkinson and co-authors became clear during subsequent personal communications with Dr Wilkinson. It was revealed that high salt and sucrose, as well a specific detergent, had been used in the purification of recombinant *Tb*NTR. More crucially, the use of only a single type of culture media from a specific supplier was found to support protein expression. Unfortunately, due to time constraints, I was not able to test expression and purification under these exact conditions.

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* Full-length *Tb*NTR was expressed mostly as an insoluble protein (this study and Hall *et al*., 2010), which prompted the use of truncated forms of the recombinant protein.

In the mean time, new information regarding *Tb*NTR became available through studies on the mitochondrial proteome of procyclic *T. brucei* (Panigrahi *et al*., 2009). *Tb*NTR had been previously shown to localise to the mitochondrion of BSF trypanosomes (Wilkinson *et al*., 2008), and the new data confirmed this localisation. Furthermore, the new study indicated that truncated forms of *Tb*NTR, which had been studied both by the Wilkinson group and myself, were missing part of the mature protein. Based on this information, I designed several alternative truncations of *Tb*NTR, one of which (*Tb*NTR$_{\Delta 54}$) was found to be enzymatically active both in bacterial lysates and in intact *E. coli* cells (using the disc diffusion method). The presence of measurable nitroreductase activity with recombinant *Tb*NTR$_{\Delta 54}$ enabled me to determine that *Tb*NTR$_{\Delta 54}$ nitroreductase activity was abolished by the presence of an affinity His_6 tag at the N-terminus, whereas a similar tag at the C-terminus was better tolerated. In addition, the recombinant protein was not amenable to purification and was found to be insoluble by gel filtration and high-force (100,000 x *g*, 1 h) centrifugation. A number of detergents were not able to solubilise *Tb*NTR$_{\Delta 54}$, with the exception of SDS and n-dodecylphosphocholine, incubation with both of which resulted in loss of nitroreductase activity. Finally, partial retention of *Tb*NTR$_{\Delta 54}$ in solution by urea and alkaline Na_2CO_3 suggested that *Tb*NTR$_{\Delta 54}$ was membrane associated. Based on these findings, the work of Wilkinson and colleagues can be questioned in terms of their recombinant *Tb*NTR, which was shorter than the mature native protein, was tagged at the N-terminus and most importantly, was not proven to be entirely soluble by any qualitative technique.

As is usually the case in research, the current study of recombinant *Tb*NTR has posed more questions than answers. For example, it remains to be established if Ser55 is the first amino acid of the processed mature *Tb*NTR in *T. brucei*. If not, what is the true start site of the native protein? Thus far, attempts to isolate *Tb*NTR from BSF *T. brucei* by two different methods have come to a halt due to technical challenges. Are the first

few amino acids of the mature protein important for enzyme activity, or are there shorter forms of *Tb*NTR that are equally active? How do changes in the length of *Tb*NTR, especially at the N-terminus, affect the protein solubility? Is it possible to obtain soluble, and at the same time enzymatically active, recombinant *Tb*NTR or is membrane association required for nitroreductase activity? My expression studies with $TbNTR_{\Delta 54}$ using cell- and membrane-free systems were inconclusive in answering this question. Moreover, the only mild detergent (n-dodecylphosphocholine) that was found to affect the solubility of *Tb*NTR was at the expense of the enzyme activity. Could the interaction of *Tb*NTR with this detergent, which differs from phospholipids only by the presence of one and not two hydrophobic chains, provide any clues to the nature of the membrane-*Tb*NTR interaction? Finally, the most enigmatic question of all – what is the real biological function of *Tb*NTR in trypanosomes?

It is not easy to speculate on the answer to this final question, especially given that the physiological function or substrate(s) has not been identified for the closest *Tb*NTR homologue – the bacterial type I nitroreductases of group B. Although *Ec*NfsB (McCalla *et al*., 1978) and similar nitroreductases have been investigated for their ability to reduce a broad range of nitroaromatic compounds, these chemicals are predominantly xenobiotics, such as synthetic antibiotics (nitrofurazone), toxic (dinoseb) or explosive (trinitrotoluene) environmental pollutants. However, a few more relevant, physiological substrates, including quinones and flavin co-factors, can also be reduced by *Ec*NfsB-like nitroreductases (Terpe, 2003). Recombinant trypanosomal NTRs have been shown to share some nitroaromatic substrates with *Ec*NfsB, most notably nitrofurazone and CB1954 (this work; Bot *et al*., 2010; Wilkinson *et al*., 2008), and it is tempting to speculate that they may also be able to reduce quinones. Combined with the mitochondrial localisation of *Tb*NTR, reduction of quinones would suggest a role in maintenance of the mitochondrial redox balance or even in respiration/oxidative

phosphorylation. Here, I propose that *Tb*NTR may be identical to a rotenone-insensitive NADH-dehydrogenase, which was recently isolated from procyclic *T. brucei* and about which no sequence information is available (Fang and Beattie, 2002). The reasons for this proposal are the following. First, this alternative NADH-dehydrogenase is a 33-kDa protein, which forms 65-kDa functional dimers and binds non-covalently to FMN (Fang and Beattie, 2002). The protein size is consistent with that of *Tb*NTR, which in its mature form is less than 35.5 kDa (full-length protein; ProtParam) and more than 29.5 kDa (the shortest possible polypeptide, as identified by proteomics MS analysis; Panigrahi *et al*., 2009). As a homologue of *Ec*NfsB, *Tb*NTR would also be expected to form dimers, and sequence alignment has shown that the trypanosomal protein has conserved the key residues required for FMN-binding. Second, the alternative NADH-dehydrogenase is "loosely bound to the inner mitochondrial membrane" (Fang and Beattie, 2002), which is consistent with the findings that *Tb*NTR is most probably membrane-associated (this study) and localised to the mitochondrion in *T. brucei* (Panigrahi *et al*., 2009; Wilkinson *et al*., 2008). Finally, the rotenone-insensitive NADH-dehydrogenase was shown to reduce ubiquinone via one-electron transfer, preferentially using NADH as the electron donor (Fang and Beattie, 2002). Although the role of this alternative cytochrome-lacking reductase may be minor in procyclic *T. brucei* that have a fully functional cytochrome-dependent respiratory chain, the presence of such an NADH-dehydrogenase may be crucial for maintaining the NADH/NAD^+ balance in BSF mitochondria (for more details, please refer to reviews by Besteiro *et al*., 2005 and Ginger, 2006). This crucial role of the 33-kDa rotenone-insensitive NADH-dehydrogenase is again consistent with *Tb*NTR, which was proposed to be essential in BSF *T. brucei* (Wilkinson *et al*., 2008). Therefore, it will be a priority for future investigations to establish whether *Tb*NTR and this alternative *T. brucei* NADH-dehydrogenase are in fact the same protein.

5.2.3. Future work with *Tb*NTR

In order to determine whether the novel 33-kDa NADH-dehydrogenase corresponds to *Tb*NTR, future efforts should be targeted at the isolation of this NADH-dehydrogenase from procyclic *T. brucei* according to the published procedure (Fang and Beattie, 2002). The isolated protein should be fully characterised in terms of its protein sequence and enzyme activity, including its ability to reduce nifurtimox and other nitroaromatic compounds. If this protein is indeed *Tb*NTR, knowledge of its sequence will help to determine the elusive true start site of mature *Tb*NTR. If the two proteins happen to be unrelated, isolation of mature *Tb*NTR ought nevertheless be pursued, using an immunoprecipitation protocol that takes into account the technical issues with the current pull-down method. It is anticipated that knowledge of the correct length of mature *Tb*NTR will address some of the issues regarding the solubility and enzymatic activity of the recombinantly expressed protein. An alternative method for improving the solubility of recombinant *Tb*NTR has been suggested by a theoretical model of the tertiary *Tb*NTR structure (**Figure 5.1**, done with the kind assistance of Dr Scott Cameron), which was based on the closest homologue of *Tb*NTR with a published crystal structure – the NADH oxidase *Tt*NOX (Hecht *et al*., 1995). According to this model, a particular stretch of amino acids in *Tb*NTR (circled on the figure, not present in *Tt*NOX) may be responsible for membrane association. Therefore, if *Tb*NTR is engineered without this segment, the recombinant protein may have an improved solubility and will hopefully retain its enzyme activity. Alternatively, substitutions of some hydrophobic residues within this segment with suitable hydrophilic counterparts may aid solubility. Should all future efforts to express recombinant *Tb*NTR result in insoluble protein, it will still be possible to assess the activity of the membrane-associated recombinant protein in intact *E. coli* cells, using an optimised and adapted

disc diffusion technique. Finally, the activity of the native (through knock-out and overexpression) and recombinant *Tb*NTR with fexinidazole should be investigated.

5.3. Mechanisms of (cross-)resistance to nitro pro-drugs in *T. brucei*

Resistance to nitroaromatic drugs occurs naturally in the South American trypanosome (Filardi and Brener, 1987), and there is some evidence to suggest that this may also be the case in African trypanosomes (Likeufack *et al*., 2006; Maina *et al*., 2007; Pépin and Milord, 1994). To determine what mechanisms may lead to such resistance in *T. brucei*, I have generated an *in vitro* nifurtimox-resistant *T. brucei* line, designated NfxR. Clones derived from this line (NfxR1 and NfxR2) were resistant not only to nifurtimox but also to several other nitroaromatic derivatives, including fexinidazole and its metabolites, the sulfone and sulfoxide. Conversely, when resistance was generated to fexinidazole, the resulting FxR cells were cross-resistant to nifurtimox, although the resistance factors to nifurtimox and fexinidazole were not reciprocal to those determined for NfxR. Overall, these findings are in agreement with other studies, which have demonstrated that both naturally-derived and laboratory-generated lines of *T. cruzi* and of *T. brucei* can be cross-resistant to various nitroaromatic drugs (Enanga *et al*., 2003; Filardi and Brener, 1987; Murta and Romanha, 1998; Wilkinson *et al*., 2008). The presence of such cross-resistance strongly indicates that nitroaromatic compounds share common mechanisms of action and of resistance. However, there may also be some resistance mechanisms that affect only a certain type of nitrodrug, as demonstrated here by the non-reciprocal resistance to the nitrofuran nifurtimox and the nitroimidazole fexinidazole.

5.3.1. Potential mechanisms of nitrodrug resistance in *T. brucei*

The mechanisms that may lead to drug resistance in trypanosomatids can be classified into three categories – mechanisms that lower the drug levels within the cell,

mechanisms that affect the main enzymes interacting with the drug (in the case of pro-drugs – the main activating proteins), and general defence and repair mechanisms (Croft *et al*., 2006).

Alterations in pro-drug activating enzymes in nitrodrug-resistant T. brucei

Bearing in mind that nitroaromatic pro-drugs are thought to require enzymatic activation as a prerequisite for biological activity, it would be obvious to consider loss of function (through either gene deletion or mutations) of the activating enzyme as the first possible mechanism of resistance. A precedent of gene loss of *NTR* – now thought to be the main activator of nitroaromatic drugs in trypanosomes, has already been described in laboratory-generated nifurtimox-resistant *T. cruzi* (Wilkinson *et al*., 2008). In this study, both deletion of one *TbNTR* copy and a substitution mutation in the remaining gene copy were found to arise in the process of generating resistance to nifurtimox, but surprisingly not to fexinidazole. These results, which were initially questioned due to the quality of the Southern hybridisation analysis, have now been confirmed by genome sequencing[*] of the parental wild-type cells and of NfxR- and FxR-derived clones (unpublished data). However, loss of a single *TbNTR* copy can account only for 1.6- to 3-fold resistance to nifurtimox (as shown in SKO *T. brucei* lines; Wilkinson *et al*., 2008 and this study), whereas the detected mutation can be disregarded as a resistance factor, because the resulting mutant protein (*Tb*NTR$_{Val106Ile}$) retained its nitroreductase activity when expressed in both *T. brucei* and *E. coli*. These findings clearly indicate that other mechanisms, in addition to loss or modification of *TbNTR*, must contribute to the 8-fold resistance to nifurtimox in the NfxR cell line. Furthermore, the above results suggest that resistance to fexinidazole can also be generated through mechanisms that do not affect the activation of this pro-drug by

[*] In collaboration with Matthew Berriman and the Wellcome Trust Sanger Institute

NTR. (It should be noted that activation of fexinidazole by NTR has not been investigated in the current study on *T. brucei* due to time constraints. However, studies in the related kinetoplastid *Leishmania* have confirmed the role of this enzyme in fexinidazole activation; Wyllie and Fairlamb, unpublished results).

Mechanisms leading to reduced intracellular levels of nitrodrugs

Other mechanisms that may lead to drug resistance in trypanosomes cause reduced exposure to the drug by decreasing the intracellular drug concentration. This can be achieved either via reducing the rate of drug uptake (for example by down-regulation of a transporter) or via increasing the rate of drug efflux. Alternatively, sequestration of the drug may also provide a protective effect and thus contribute to drug resistance (Croft *et al*., 2006). To my knowledge, there are no studies on the uptake of nifurtimox in *T. brucei*. However, in *T. cruzi*, this lipophilic drug has been suggested to enter the cells via passive diffusion (Tsuhako *et al*., 1991). In *T. brucei*, the related nitrodrug megazol is also taken up primarily via passive diffusion, even though megazol has a recognition motif for the P2 amino-purine transporter (Barrett *et al*., 2000). The uptake of both nifurtimox and megazol has been suggested to occur in two phases – a rapid first phase of equilibration between the drug concentration in the cell and the extracellular media, followed by a much slower phase that is driven by "removal" of the intracellular drug, either through drug metabolism or sequestration (Barrett *et al*., 2000). Our preliminary studies on uptake of radiolabelled nifurtimox in *T. brucei* seem to support this biphasic model, but shortage of the radiolabelled drug has prevented more thorough investigations. Should my findings be confirmed by future studies, active pro-drug uptake could be excluded as a mechanism of nitrodrug resistance due to the lack of a dedicated transporter, although the second phase of the passive diffusion mechanism may still be influenced by reduced metabolism or reduced drug sequestration.

In trypanosomes, both drug sequestration (to an intracellular compartment) and efflux (to the extracellular media) are thought to be facilitated by members of the ABC transporter family, known as P-glycoproteins or multidrug resistance associated proteins (Klokouzas *et al*., 2003). Overexpression of different ABC transporters in *T. brucei* has been shown to cause resistance to melarsoprol and suramin (Shahi *et al*., 2002). A similar mechanism of resistance to nifurtimox also appears to operate in a strain of *T. cruzi*, because the resistance phenotype of these cells could be reversed by the calcium-channel inhibitor verapamil (Neal *et al*., 1989). However, other studies have shown that non-toxic concentrations of verapamil did not affect the sensitivity of *T. brucei* to two drugs active against animal trypanosomiasis (Kaminsky and Zweygarth, 1991), and more importantly to the nitrodrug megazol (Enanga *et al*., 2003). Based on these last reports, it would seem less likely than that efflux and/or sequestration are involved in nitrodrug resistance in the African trypanosome.

Antioxidant and damage-repair mechanisms in nitrodrug resistance

Cell defence mechanisms may help to protect the cell or reverse the damage caused by nitrodrugs, and therefore up-regulation of these mechanisms has the potential to enhance drug resistance. A link between nitrodrug resistance and oxidant defence has been implied in several benznidazole-resistant strains of *T. cruzi*, which were found to have increased levels of the antioxidant enzymes tryparedoxin peroxidase (TryP) and iron superoxide dismutase (Andrade *et al*., 2008). Both nitrofurans and nitroimidazoles have been shown to deplete reduced trypanothione in *T. cruzi* (Maya *et al*., 2003). Conversely, depletion of trypanothione by RNA interference of its synthetase, TryS, was found to increase the sensitivity of *T. brucei* to nifurtimox (Ariyanayagam *et al*., 2005). Just as decreased levels of trypanothione are associated with increased nitrodrug sensitivity, it would be expected that increase of the intracellular trypanothione content

would provide a potential mechanism of nitrodrug resistance. However, unpublished studies performed by Dr Susan Wyllie in our laboratory, have found no significant difference in the levels of trypanothione and total thiols between our nitrodrug-resistant (NfxR and FxR) and -sensitive *T. brucei* cell lines. Similar results have also been obtained in laboratory-generated *T. brucei* resistant to megazol (Enanga *et al*., 2003).

Trypanothione is a molecule central to the antioxidant defence mechanisms in trypanosomes (Ariyanayagam and Fairlamb, 2001). Combined with the above studies on TryP and superoxide dismutase, this would seem to imply that nitroaromatic pro-drugs can act via inducing oxidative stress (for example, through redox cycling) and that counteracting such stress would provide a mechanism of resistance. However, trypanothione can also provide protection from chemical stress, induced by drugs (Mukhopadhyay *et al*., 1996), metabolites (Vickers *et al*., 2004) and xenobiotics (Fairlamb and Cerami, 1992; Vickers and Fairlamb, 2004). Moreover, nitrofurans are no longer thought to act via production of reactive oxygen species in trypanosomes (Boiani *et al*., 2010; Hall *et al*., 2011), and it is believed that nitroimidazole metabolites act via damage to DNA and other macromolecules (Diaz-de-Toranzo *et al*., 1988; Enanga *et al*., 2003). In fact, depletion of the reduced trypanothione pool in *T. cruzi* was thought to occur not via oxidation, but by formation of trypanothione-drug conjugates (Maya *et al*., 2003). Therefore, up-regulation of oxidative defences may not necessarily present a plausible mechanism of resistance in trypanosomes. Elevated levels of low molecular weight thiols can also be excluded as a mechanism for nitrodrug resistance in NfxR and FxR cells, although it is possible that other defence systems, such as DNA repair mechanisms, are involved.

In conclusion, conventional methods have not been able to identify all of the resistance mechanisms operational in our NfxR and FxR cell lines. It is hoped that more detailed examinations of their respective genome sequences will reveal additional

insights into to nature of this resistance, and potentially into the mechanisms of action of nifurtimox and especially of fexinidazole.

5.3.2. Future studies with nitrodrug-resistant *T. brucei*

The genome sequence of our nifurtimox- and fexinidazole-resistant cells is currently under analysis and is expected to facilitate the identification of the potential mechanisms involved in nitrodrug resistance. The only mechanism identified so far is partial loss of *TbNTR* in NfxR-derived clonal lines. This finding is consistent with the resistance profile of NfxR1 and NfxR2, which were cross-resistant to nitrofurazone and CB1954 – two known substrates of *Tb*NTR (Bot *et al*., 2010) and *Ec*NfsB (Anlezark *et al*., 1992), but were not resistant to PA-824 – a nitrodrug that is activated by *Mycobacterium tuberculosis* deazaflavin reductase not found in *T. brucei* (Singh *et al*., 2008). It should be determined whether FxR cells, which have retained their *TbNTR* genes and which have been tested only with nifurtimox, have a similar cross-resistance profile to other nitroaromatic pro-drugs. It would also be very important to establish whether *Tb*NTR is expressed to the same levels in FxR as in the parental wild-type cells. Currently there is neither a suitable antibody that can detect *Tb*NTR in *T. brucei* lysates, nor a sufficiently sensitive enzyme assay to measure the levels of nitroreductase activity in these lysates. However, changes in NTR expression levels might be detected by comparing mRNA levels between the nitrodrug-resistant and -sensitive cells, for example by Northern blot analysis (Sienkiewicz *et al*., 2008) or by real-time PCR (Mejia-Jaramillo *et al*., 2011). It might be of interest to determine at what stage in the selection process the loss of *TbNTR* occurred in the NfxR cell line. It has been proposed that this may have happened during selection with 38 µM nifurtimox, since this step was not required to select for fexinidazole resistance (the cells survived an increase from 25 µM to 50 µM fexinidazole). Another area of research that cannot necessarily be

addressed by reference to the genome sequence is the rate of nifurtimox uptake by NfxR and FxR compared to that in the parental wild-type *T. brucei*. However, such studies will require synthesis of isotopically labelled drug. Finally, the intracellular levels of trypanothione under normal culture conditions appear to be identical between the nitrodrug-resistant and -sensitive *T. brucei*, but there is a possibility that the thiol levels of these cell lines might be affected in a different way under nitrodrug exposure. Therefore, determination of thiol levels in NfxR and FxR cells under stress conditions would form an additional subject for future investigation.

5.4. Clinical implications of nitrodrug resistance in *T. brucei*

5.4.1. Resistance to nifurtimox

In a clinical setting, drug resistance can occur either naturally, such as the inherent lack of susceptibility of *T. b. rhodesiense* to eflornithine (Fries and Fairlamb, 2003), or under selective drug pressure, such as resistance to antimonials in *Leishmania*, which is proposed to have arisen due to drug misuse (Croft *et al*., 2006). Regarding nitroaromatic drugs, naturally-occurring resistance to nifurtimox is now a well-established fact in *T. cruzi* (Filardi and Brener, 1987). The high failure-to-cure rates (up to 70%), established when nifurtimox was initially tested against *T. brucei*, have led to concerns that natural resistance may also be a factor in the African trypanosome (Pépin and Milord, 1994). However, when nifurtimox was first developed for use against Chagas' disease, it was suggested that in a given strain of *T. cruzi*, it was not possible to select for "complete resistance" to nifurtimox *in vivo* over the course of two years (Haberkorn and Gonnert, 1972). A more recent study has shown that resistance in *T. cruzi* can be selected under nifurtimox pressure *in vitro* within 8 months (Wilkinson *et al*., 2008), and a similar study in *T. brucei* demonstrated generation of *in vitro* resistance to megazol within 6 months (Enanga *et al*., 2003). However, these two studies have not determined the

clinical relevance of their resistant lines. In the current work, I have reported on the *in vitro* generation of nifurtimox resistance in *T. brucei*, without loss of virulence in the resulting NfxR1 cells, and at clinically relevant levels as demonstrated by the failure of nifurtimox to cure NfxR1-infected mice even at near-lethal doses. Moreover, NfxR1 *T. brucei* were found to be cross-resistant to fexinidazole *in vivo*. At least 2 out of 5 NfxR1-infected mice relapsed after a 4-day fexinidazole treatment at 200 mg kg^{-1} day^{-1} (the highest dose that does not cause adverse effects; Torreele *et al*., 2010), whereas the number of relapses was double (4 out of 5) at 100 mg kg^{-1} day^{-1} (the curative dose in the acute model of infection; Torreele *et al*., 2010). These findings suggest that clinically-relevant resistance to nifurtimox can be generated relatively easy in BSF trypanosomes. Should such resistance occur in the field (if it is has not already), it will not only compromise the widespread implementation of NECT, but may also preclude any plans to introduce fexinidazole (and potentially other nitroaromatics) as a treatment against HAT. As previously discussed (Sokolova *et al*., 2010), combination therapy provides an effective strategy to reduce the likelihood of emerging drug resistance. Bearing this in mind, the findings of this current study strongly support the notion that nifurtimox should continue to be used against sleeping sickness only in drug combination therapy, such as NECT.

The rationale behind introducing combination therapy for a given indication is rarely prevention of emerging resistance, as exemplified by the first clinical trial to suggest the usefulness of NECT against HAT (Priotto *et al*., 2006). The anticipation at the onset of this clinical trial was that combination therapy would allow drugs to be used at decreased doses without loss of efficacy, but potentially with minimised toxicity. In the case of NECT, the use of lower eflornithine doses has also resulted in great reduction of the treatment cost (Yun *et al*., 2010). Maintaining the efficacy at lower drug doses would normally be expected if the two drugs, used in the combination,

have a synergistic or at least an additive effect. However, a recent study on drug interaction *in vitro* has reported that nifurtimox and eflornithine may be antagonistic (Vincent *et al*., 2010). This result is highly surprising, given that the efficacy of the nifurtimox-eflornithine combination (96.5%) was similar to, if not higher than that of eflornithine monotherapy (91.6%; Priotto *et al*., 2009), and much greater than previously reported for nifurtimox monotherapy (30 – 87%; Bouteille *et al*., 2003). In fact, initial findings in our laboratory (Fu, Wyllie and Fairlamb, unpublished) suggest that the interaction between nifurtimox and eflornithine is somewhat complex, and, although it appears to be antagonistic at low eflornithine concentrations, it may become synergistic at higher levels of eflornithine. One factor that is not taken into account in both of these studies is the effect of time. During *in vitro* determinations of drug interactions (such as by Vincent *et al*., 2010), the two compounds are usually tested simultaneously. It is possible that for a synergistic effect to occur eflornithine needs to be given first (or at much higher doses), so that sufficient reduction of trypanothione levels can occur (Fairlamb *et al*., 1987), before the cells become more susceptible to nifurtimox (Ariyanayagam *et al*., 2005). Finally, host-related factors, which cannot be accounted for during *in vitro* studies, such as drug metabolism or the host immune system, may also influence the outcome of the combination therapy. For example, the curative effect of eflornithine against HAT has been shown to depend on an effective immune response (Bitonti *et al*., 1986), whereas nifurtimox is known to be metabolised very rapidly (half-life in humans of only 3 h; Paulos *et al*., 1989) and extensively (<1% excreted unchanged in urine; Medenwald *et al*., 1972). Because the nature of these metabolites remains unknown, their effect on trypanosomes as well as their interaction with eflornithine cannot be determined. Therefore, more thorough investigations will be required to determine the type of drug interactions (synergism or antagonism) between nifurtimox and eflornithine, which should form the basis of our future studies. Initial

efforts should elaborate on the findings of dose-dependent synergism between the two drugs *in vitro*. An additional area to be explored may include the identification of nifurtimox metabolites (potentially in collaboration with the DMPK group) and their efficacy against trypanosomes. The starting point for such studies could be the related drug nitrofurazone, some metabolites of which have been identified as the hydroxylamino and amino derivatives, 4-cyano-2-oxobutyraldehyde semicarbazone and two other coloured compounds of unknown structure (Miura and Reckendorf, 1967). Interestingly, the 4-cyano metabolite of nitrofurazone corresponds to the "saturated open nitrile chain" of nifurtimox, derived after reduction by *Tb*NTR (Hall *et al*., 2011). If similar metabolites are detected for nifurtimox *in vivo*, this should not only help to uncover the effect of these compounds on trypanosomes, but may also explain the relatively high toxicity of nifurtimox and other nitrofurans in mammals.

5.4.2. Resistance to fexinidazole

At the pinnacle of nitroaromatic drug discovery in the 1960s and '70s, it was noted that the *in vitro* potency of nitrodrugs does not necessarily correlate with their *in vivo* efficacy, due to host-related factors, such as drug absorption, metabolism and excretion (Grunberg and Titsworth, 1973). This holds true also for nifurtimox and fexinidazole, which are active against *T. brucei* over a similar concentration range *in vitro* (this study and Torreele *et al*., 2010). However, the efficacy of fexinidazole *in vivo* is higher than that of nifurtimox, as illustrated by the 99% effective dose of fexinidazole against *T. brucei* S427 in mice (~38 mg kg^{-1} day^{-1} over 4 days; this study), which would only result in ~50% cure if applied for nifurtimox under the same experimental conditions (Fairlamb *et al*., 1992). In part, this difference in activity between fexinidazole and nifurtimox *in vivo* could be explained by drug metabolism. Although both compounds are metabolised very rapidly in mammals (Duhm *et al*., 1972; Paulos *et al*., 1989;

Torreele *et al*., 2010), there is no information on the metabolites of nifurtimox, whereas at least two fexinidazole metabolites (the sulfone and sulfoxide; Winkelmann and Raether, 1978) have been identified. Both of these metabolites have similar potency to fexinidazole *in vitro* against all three subspecies of *T. brucei* (this study and Torreele *et al*., 2010). Moreover, the metabolites of fexinidazole reach blood concentrations higher than their respective EC_{99} values against *T. brucei*, and maintain these high concentrations over several hours both after a single (this study) or a 5-day (Torreele *et al*., 2010) oral exposure to fexinidazole in mice. Therefore, the combined activity of fexinidazole and its metabolites is likely to account for the high *in vivo* efficacy of this drug. Fexinidazole is not only more effective than nifurtimox against trypanosomes, but it is also much better tolerated in mammals as demonstrated in the current and other studies (no effect dose was 25 – 30 mg kg^{-1} for nifurtimox, Hoffmann, 1972; and 200 mg kg^{-1} for fexinidazole, Torreele *et al*., 2010).

Collectively, all of the above studies indicate that, unlike nifurtimox, fexinidazole can be safely administered at doses much higher than needed for an effective cure of African trypanosomiasis. The broad therapeutic window of fexinidazole may lead to the impression that relative to nifurtimox, it would be much more difficult to select for fexinidazole resistance *in vivo* or in a field setting. However, there are several arguments to the contrary. In the current work, I have demonstrated that it is just as easy to select for fexinidazole resistance *in vitro* as it is for nifurtimox. The resulting resistant cell line FxR was 10-fold less susceptible to fexinidazole than the parental wild-type cells. However, FxR have the potential to become even more resistant, if the cells were to lose a copy of the nitrodrug activating enzyme *Tb*NTR. Furthermore, although the sensitivity of FxR to fexinidazole has not yet been determined *in vivo*, comparison of the fexinidazole sensitivity of FxR and NfxR cells from *in vitro* studies indicates that FxR cells may be refractory to fexinidazole

treatment. The EC_{99} for this drug against FxR2 (670 μM) was less than 2-fold lower than that against NfxR1 (1080 μM), which were resistant to the highest test concentration of fexinidazole *in vivo*. Theoretically, since two out of five NfxR1-infected mice relapsed after treatment with 200 mg kg^{-1} fexinidazole in our acute model of infection, at least one out five FxR2-infected mice would be expected to relapse in a potential equivalent experiment. Additionally, our pharmacological studies in a mouse model indicate that the maximum achievable total concentration of fexinidazole and its metabolites in plasma is ~240 μM (8 h after a single oral dose of 200 mg kg^{-1}), whereas the free maximum concentration of drug, not bound to plasma proteins and therefore available to interact with the parasites, was ~180 μM. The maximum achievable total and free concentrations of this drug in the brain, which is affected during the second disease stage, are expected to be even lower, as studies in the rat demonstrated a total "brain-to-blood concentration ratio of fexinidazole and its metabolites of 0.4 – 0.6" (Torreele *et al*., 2010). Even though no direct comparison can be made between these *in vivo* studies and the sensitivity of *T. brucei* to fexinidazole *in vitro*, our data seems to suggest that FxR cells might be able to survive at drug concentrations much higher that those achievable in the plasma and in the brain. If these expectations are confirmed through subsequent studies, they will prove that it is possible to generate clinically relevant resistance in *T. brucei* not only to nifurtimox but also to fexinidazole. In addition, early studies with fexinidazole in a chronic mouse model of sleeping sickness have already demonstrated that this drug is not always effective, even at high doses (Jennings and Urquhart, 1983). Therefore, the current plans to introduce fexinidazole as a short-course monotherapy for sleeping sickness in an oral formulation (Torreele *et al*., 2010) may need to be revisited to include proper investigations on the potential of African trypanosomes to develop *in vivo* resistance to this drug. Finally, it should be noted that although orally administered drugs may be much less distressing for the

patient and less costly because no or minimal hospitalisation is required, they are more susceptible to patient non-compliance (Croft *et al*., 2006). Based on these arguments, it would be highly advisable to consider the use of fexinidazole not as a monotherapy, but as a combination therapy, similar to that of nifurtimox. Historical experiments with fexinidazole have suggested that combinations of this drug and other antitrypanosomal agents have a very high *in vivo* efficacy against *T. brucei* (Jennings, 1993). The same study had already demonstrated the high efficacy of fexinidazole in combination and eflornithine for the treatment of second-stage trypanosomiasis. Future efforts should be targeted at identifying other suitable partner drugs for fexinidazole. These partner drugs may include another new class currently under investigation for its antitrypanosomal activity – the oxaboroles (Barrett, 2010).

5.5. Conclusions

Some of the key findings of this current work have indicated that resistance to nifurtimox may be relatively easy to arise in the field in the African trypanosome. Due the small therapeutic index of nifurtimox, even a small decrease in the trypanosome sensitivity to nifurtimox would be expected to change the clinical outcome from cure to relapse. The therapeutic index of fexinidazole is much broader than that of nifurtimox and doses higher than those required for cure can be safely administered. Nonetheless, I speculate that resistance to this drug may be just as easy to arise in *T. brucei* as is for nifurtimox. One of the key mechanisms that can lead to such nitrodrug resistance in trypanosomes is partial loss of *TbNTR*, which I have confirmed to be a main activating enzyme of nitro pro-drugs in *T. brucei*. My attempts to purify recombinant *Tb*NTR for further detailed analysis and eventually for crystallographic studies have been hampered by the insoluble nature of this protein, which was found to be membrane associated. Although *Tb*NTR could be purified in a denatured form, which has been used to raise

anti-*Tb*NTR polyclonal antibodies, it is arguable whether recombinant *Tb*NTR can be obtained in a soluble and at the same time enzymatically active form. Undoubtedly, down-regulation of *Tb*NTR presents an important mechanism of resistance to nitroaromatic drugs and presents a common mechanism, which can lead to cross-resistance to all nitroaromatic pro-drugs that act as *Tb*NTR substrates. However, partial loss of this protein causes only a low-level nitrodrug resistance in *T. brucei*. Therefore, multiple mechanisms of resistance, waiting to be discovered, must be operational in these parasites. Because of the shared mechanisms of action, activation and resistance between nitro pro-drugs, emerging resistance to one drug can affect multiple compounds of the same chemical class. If such cross-resistance arises in the field in the African trypanosome, this would have devastating consequences not only on the existing chemotherapeutic options, such as NECT, but on all future efforts to introduce other nitroaromatics, including fexinidazole, for the treatment of HAT. Therefore, the use of combination chemotherapy may be essential to protect both nifurtimox and fexinidazole against the emergence of resistance. From the findings in this study, it could be concluded that effective policies should be introduced to ensure compliance with the use of nifurtimox as a combination therapy and that partner drugs (for example eflornithine) should be sought for fexinidazole, before this drug is introduced against sleeping sickness.

Reference list

Medical Insects and Arachnids (1993). R.P. Lane and R.W. Crosskey, eds. (London: Chapman & Hall), pp. 1-723.

Human African trypanosomiasis (sleeping sickness): epidemiological update (2006). *Wkly. Epidemiol. Rec. 81*, 71-80.

Abolarin, M.O., Evans, D.A., Tovey, D.G. and Ormerod, W.E. (1982). Cryptic stage of sleeping sickness trypanosome developing in choroid plexus epithelial cells. *BMJ 285*, 1380-1382.

Agarwal, P. and Berglund, K.A. (2004). Effect of polymeric additives on calcium carbonate crystallization as monitored by nephelometry. *Cryst. Growth Des. 4*, 479-483.

Andrade, H.M., Murta, S.M.F., Chapeaurouge, A., Perales, J., Nirde, P. and Romanha, A.J. (2008). Proteomic analysis of *Trypanosoma cruzi* resistance to benznidazole. *J. Proteome Res. 7*, 2357-2367.

Anlezark, G.M., Melton, R.G., Sherwood, R.F., Coles, B., Friedlos, F. and Knox, R.J. (1992). The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) .1. Purification and properties of a nitroreductase enzyme from *Escherichia coli* - A potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). *Biochem. Pharmacol. 44*, 2289-2295.

Ariyanayagam, M.R. and Fairlamb, A.H. (2001). Ovothiol and trypanothione as antioxidants in trypanosomatids. *Mol. Biochem. Parasitol. 115*, 189-198.

Ariyanayagam, M.R., Oza, S.L., Guther, M.L. and Fairlamb, A.H. (2005). Phenotypic analysis of trypanothione synthetase knockdown in the African trypanosome. *Biochem. J. 391*, 425-432.

Baker, N., Alsford, S. and Horn, D. (2010). Genome-wide RNAi screens in African trypanosomes identify the nifurtimox activator NTR and the eflornithine transporter AAT6. *Mol. Biochem. Parasitol. 176*, 55-57.

Balmer, O., Beadell, J.S., Gibson, W. and Caccone, A. (2011). Phylogeography and Taxonomy of *Trypanosoma brucei*. *PLoS Negl. Trop. Dis. 5*.

Barrett, M.P. (2010). Potential new drugs for human African trypanosomiasis: some progress at last. *Curr. Opin. Infect. Dis. 23*, 603-608.

Barrett, M.P., Fairlamb, A.H., Rousseau, B., Chauviere, G. and Perie, J. (2000). Uptake of the nitroimidazole drug megazol by African trypanosomes. *Biochem. Pharmacol. 59*, 615-620.

Batchelor, N.A., Atkinson, P.M., Gething, P.W., Picozzi, K., Fevre, E.M., Kakembo, A.S.L. and Welburn, S.C. (2009). Spatial predictions of Rhodesian Human African Trypanosomiasis (sleeping sickness) prevalence in Kaberamaido and Dokolo, two newly affected districts of Uganda. *PLoS Negl. Trop. Dis. 3*, e12.

Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol. 45*, 493-496.

Beckers, A., Wery, M., Vanmarck, E. and Gigase, P. (1981). Experimental infections of laboratory rodents with recently isolated stocks of *Trypanosoma brucei gambiense* .1. parasitological investigations. *Z. Parasitenkd. -Parasitol. Res. 64*, 285-296.

Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D.C., Lennard, N.J., Caler, E., Hamlin, N.E., Haas, B., Bohme, U., Hannick, L., Aslett, M.A., Shallom, J., Marcello, L., Hou, L., Wickstead, B., Alsmark, U.C., Arrowsmith, C., Atkin, R.J., Barron, A.J., Bringaud, F., Brooks, K., Carrington, M., Cherevach, I., Chillingworth, T.J., Churcher, C., Clark, L.N., Corton, C.H., Cronin, A., Davies, R.M., Doggett, J., Djikeng, A., Feldblyum, T., Field, M.C., Fraser, A., Goodhead, I., Hance, Z., Harper, D., Harris, B.R., Hauser, H., Hostetler, J., Ivens, A., Jagels, K., Johnson, D., Johnson, J., Jones, K., Kerhornou, A.X., Koo, H., Larke, N., Landfear, S., Larkin, C., Leech, V., Line, A., Lord, A., MacLeod, A., Mooney, P.J., Moule, S., Martin, D.M., Morgan, G.W., Mungall, K., Norbertczak, H., Ormond, D., Pai, G., Peacock, C.S., Peterson, J., Quail, M.A., Rabbinowitsch, E., Rajandream, M.A., Reitter, C., Salzberg, S.L., Sanders, M., Schobel, S., Sharp, S., Simmonds, M., Simpson, A.J., Tallon, L., Turner, C.M., Tait, A., Tivey, A.R., Van Aken, S., Walker, D., Wanless, D., Wang, S., White, B., White, O., Whitehead, S., Woodward, J., Wortman, J., Adams, M.D., Embley, T.M., Gull, K., Ullu, E., Barry, J.D., Fairlamb, A.H., Opperdoes, F., Barrell, B.G., Donelson, J.E., Hall, N., Fraser, C.M., Melville, S.E. and El Sayed, N.M. (2005). The Genome of the African Trypanosome *Trypanosoma brucei*. *Science 309*, 416-422.

Besteiro, S., Barrett, M.P., Riviere, L. and Bringaud, F. (2005). Energy generation in insect stages of *Trypanosoma brucei*: metabolism in flux. *Trends Parasitol. 21*, 185-191.

Bevan, C.D. and Lloyd, R.S. (2000). A high-throughput screening method for the determination of aqueous drug solubility using laser nephelometry in microtiter plates. *Anal. Chem. 72*, 1781-1787.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res. 7*, 1513-1523.

Bitonti, A.J., McCann, P.P. and Sjoerdsma, A. (1986). Necessity of antibody response in the treatment of African trypanosomiasis with α-difluoromethylornithine. *Biochem. Pharmacol. 35*, 331-334.

Blumenstiel, K., Schoneck, R., Yardley, V., Croft, S.L. and Krauth-Siegel, R.L. (1999). Nitrofuran drugs as common subversive substrates of *Trypanosoma cruzi* lipoamide dehydrogenase and trypanothione reductase. *Biochem. Pharmacol. 58*, 1791-1799.

Boiani, M., Piacenza, L., Hernandez, P., Boiani, L., Cerecetto, H., Gonzalez, M. and Denicola, A. (2010). Mode of action of nifurtimox and *N*-oxide-containing heterocycles against *Trypanosoma cruzi*: Is oxidative stress involved? *Biochem. Pharmacol. 79*, 1736-1745.

Bot, C., Hall, B.S., Bashir, N., Taylor, M.C., Helsby, N.A. and Wilkinson, S.R. (2010). Trypanocidal Activity of Aziridinyl Nitrobenzamide Prodrugs. *Antimicrob. Agents Chemother. 54*, 4246-4252.

Bouteille, B., Oukem, O., Bisser, S. and Dumas, M. (2003). Treatment perspectives for human African trypanosomiasis. *Fundam. Clin. Pharmacol. 17*, 171-181.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248-254.

Brun, R., Blum, J., Chappuis, F. and Burri, C. (2010). Human African trypanosomiasis. *Lancet 375*, 148-159.

Brun, R., Schumacher, R., Schmid, C., Kunz, C. and Burri, C. (2001). The phenomenon of treatment failures in Human African Trypanosomiasis. *Trop. Med. Int. Health 6*, 906-914.

Burkard, G., Fragoso, C.M. and Roditi, I. (2007). Highly efficient stable transformation of bloodstream forms of *Trypanosoma brucei*. *Mol. Biochem. Parasitol. 153*, 220-223.

Burri, C. and Brun, R. (2009). Human African Trypanosomiasis. In *Manson's Tropical Diseases*, G.C. Cook and A.I. Zumla, eds. (The Netherlands: Elsevier), pp. 1307-1325.

Chappuis, F., Lima, M.A., Flevaud, L. and Ritmeijer, K. (2010). Human African Trypanosomiasis in areas without surveillance. *Emerg. Infect. Dis. 16*, 354-356.

Chappuis, F., Loutan, L., Simarro, P., Lejon, V. and Buscher, P. (2005). Options for field diagnosis of human African trypanosomiasis. *Clin. Microbiol. Rev. 18*, 133-146.

Checchi, F., Filipe, J.A., Haydon, D.T., Chandramohan, D. and Chappuis, F. (2008a). Estimates of the duration of the early and late stage of gambiense sleeping sickness. *BMC Infect. Dis. 8*, 16.

Checchi, F., Filipe, J.A.N., Barrett, M.P. and Chandramohan, D. (2008b). The natural progression of gambiense sleeping sickness: what is the evidence? *PLoS Negl. Trop. Dis. 2*, e303.

Checkley, A.M., Pepin, J., Gibson, W.C., Taylor, M.N., Jager, H.R. and Mabey, D.C. (2007). Human African trypanosomiasis: diagnosis, relapse and survival after severe melarsoprol-induced encephalopathy. *Trans. R. Soc. Trop. Med. Hyg. 101*, 523-526.

Claros, M.G. and Vincens, P. (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur. J. Biochem. 241*, 779-786.

Cole, C., Barber, J.D. and Barton, G.J. (2008). The Jpred 3 secondary structure prediction server. *Nucleic Acids Res. 36*, W197-W201.

Croft, S.L., Sundar, S. and Fairlamb, A.H. (2006). Drug Resistance in Leishmaniasis. *Clin. Microbiol. Rev. 19*, 111-126.

De Leeuw, E., Porcelli, I., Sargent, F., Palmer, T. and Berks, B.C. (2001). Membrane interactions and self-association of the TatA and TatB components of the twin-arginine translocation pathway. *FEBS Lett. 506*, 143-148.

Deborggraeve, S. and Buscher, P. (2010). Molecular diagnostics for sleeping sickness: what is the benefit for the patient? *Lancet Infect. Dis. 10*, 433-439.

Diaz-de-Toranzo, E.G., Castro, J.A., Franke de Cazzulo, B.M. and Cazzulo, J.J. (1988). Interaction of benznidazole reactive metabolites with nuclear and kinetoplastic DNA, proteins and lipids from Trypanosoma cruzi. *Experientia 44*, 880-881.

Docampo, R. (1990). Sensitivity of parasites to free radical damage by antiparasitic drugs. *Chem. Biol. Interact. 73*, 1-27.

Docampo, R. and Moreno, S.N.J. (1986). Free-radical metabolism of antiparasitic agents. *Fed. Proc. 45*, 2471-2476.

Docampo, R., Moreno, S.N.J., Stoppani, A.O.M., Leon, W., Cruz, F.S., Villalta, F. and Muniz, R.F. (1981). Mechanism of nifurtimox toxicity in different forms of Trypanosoma cruzi. *Biochem. Pharmacol. 30*, 1947-1951.

Docampo, R. and Stoppani, A.O. (1979). Generation of superoxide anion and hydrogen peroxide induced by nifurtimox in *Trypanosoma cruzi*. *Arch. Biochem. Biophys. 197*, 317-321.

Dodd, M.C., Stillman, W.B., Roys, M. and Crosby, C. (1944). The in vitro bacteriostatic action of some simple furan derivatives. *J. Pharmacol. Exp. Ther. 82*, 11-18.

Duhm, B., Maul, W., Medenwald, H., Patzschke, K. and Wegner, L.A. (1972). Investigations on pharmacokinetics of Nifurtimox-^{35}S in the rat and dog. *Arzneimittelforschung 22*, 1617-1624.

Enanga, B., Ariyanayagam, M.R., Stewart, M.L. and Barrett, M.P. (2003). Activity of megazol, a trypanocidal nitroimidazole, is associated with DNA damage. *Antimicrob. Agents Chemother. 47*, 3368-3370.

Enanga, B., Burchmore, R.J.S., Stewart, M.L. and Barrett, M.P. (2002). Sleeping sickness and the brain. *Cellular and Molecular Life Sciences 59*, 845-858.

Fairlamb, A.H. (2003). Chemotherapy of Human African Trypanosomiasis: Current and future prospects. *Trends Parasitol. 19*, 488-494.

Fairlamb, A.H., Carter, N.S., Cunningham, M. and Smith, K. (1992). Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism. *Mol. Biochem. Parasitol. 53*, 213-222.

Fairlamb, A.H. and Cerami, A. (1992). Metabolism and functions of trypanothione in the Kinetoplastida. *Annu. Rev. Microbiol. 46*, 695-729.

Fairlamb, A.H., Henderson, G.B., Bacchi, C.J. and Cerami, A. (1987). *In vivo* effects of difluoromethylornithine on trypanothione and polyamine levels in bloodstream forms of *Trypanosoma brucei*. *Mol. Biochem. Parasitol. 24*, 185-191.

Fang, J. and Beattie, D.S. (2002). Novel FMN-containing rotenone-insensitive NADH dehydrogenase from *Trypanosoma brucei* mitochondria: Isolation and characterization. *Biochemistry 41*, 3065-3072.

Fenn, K. and Matthews, K.R. (2007). The cell biology of *Trypanosoma brucei* differentiation. *Curr. Opin. Microbiol. 10*, 539-546.

Ferrer, M., Chernikova, T.N., Timmis, K.N. and Golyshin, P.N. (2004). Expression of a temperature-sensitive esterase in a novel chaperone-based *Escherichia coli* strain. *App. Environ. Micobiol. 70*, 4499-4504.

Ferrer, M., Chernikova, T.N., Yakimov, M.M., Golyshin, P.N. and Timmis, K.N. (2003). Chaperonins govern growth of *Escherichia coli* at low temperatures. *Nat. Biotechnol. 21*, 1266-1267.

Fevre, E.M., Coleman, P.G., Welburn, S.C. and Maudlin, I. (2004). Reanalyzing the 1900-1920 sleeping sickness epidemic in Uganda. *Emerg. Infect. Dis. 10*, 567-573.

Fevre, E.M., Picozzi, K., Jannin, J., Welburn, S.C. and Maudlin, I. (2006). Human African trypanosomiasis: epidemiology and control. *Adv. Parasitol. 61*, 167-221.

Filardi, L.S. and Brener, Z. (1987). Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. *Trans. R. Soc. Trop. Med. Hyg. 81*, 755-759.

Foulkes, J.R. (1996). Metronidazole and suramin combination in the treatment of arsenical refractory rhodesian sleeping sickness - A case study. *Trans. R. Soc. Trop. Med. Hyg. 90*, 422.

Frearson, J.A., Wyatt, P.A., Gilbert, I.H. and Fairlamb, A.H. (2007). Target assessment for antiparasitic drug discovery. *Trends Parasitol. 23*, 589-595.

Fries, D.S. and Fairlamb, A. H. (2003). Antiprotozoal Agents. In *Burger's Medicinal Chemistry and Drug Discovery, volume 5: Chemotherapeutic Agents*, D.J. Abraham, ed. (New York: Wiley and Sons), pp. 1033-1087.

Gamarro, F., Yu, P.L., Zhao, J., Edman, U., Greene, P.J. and Santi, D. (1995a). Erratum: *Trypanosoma brucei* dihydrofolate reductase-thymidylate synthase: Gene isolation and expression and characterization of the enzyme (Molecular and Biochemical Parasitology (1995) Vol. 72 (pp. 11-22)). *Mol. Biochem. Parasitol. 75*, 127.

Gamarro, F., Yu, P.L., Zhao, J., Edman, U., Greene, P.J. and Santi, D. (1995b). *Trypanosoma brucei* dihydrofolate reductase-thymidylate synthase: Gene isolation and expression and characterization of the enzyme. *Mol. Biochem. Parasitol. 72*, 11-22.

Ginger, M.L. (2006). Niche metabolism in parasitic protozoa. *Philos. Trans. R. Soc. Lond B Biol. Sci. 361*, 101-118.

Ginsberg, A.M., Laurenzi, M.W., Rouse, D.J., Whitney, K.D. and Spigelman, M.K. (2009). Safety, tolerability, and pharmacokinetics of PA-824 in healthy subjects. *Antimicrob. Agents Chemother. 53*, 3720-3725.

Giroud, C., Ottones, F., Coustou, V., Dacheux, D., Biteau, N., Miezan, B., Van Reet, N., Carrington, M., Doua, F. and Baltz, T. (2009). Murine models for *Trypanosoma brucei gambiense* disease progression - from silent to chronic infections and early brain tropism. *PLoS Negl. Trop. Dis. 3*.

Gray, G.D., Jennings, F.W. and Hajduk, S.L. (1982). Relapse of Monomorphic and Pleomorphic *Trypanosoma brucei* Infections in the mouse after chemotherapy. *Z. Parasitenkd. -Parasitol. Res. 67*, 137-145.

Greig, N., Wyllie, S., Patterson, S. and Fairlamb, A.H. (2009). A comparative study of methylglyoxal metabolism in trypanosomatids. *FEBS J. 276*, 376-386.

Grunberg, E. and Titsworth, E.H. (1973). Chemotherapeutic properties of heterocyclic-compounds - monocyclic compounds with 5-membered rings. *Annu. Rev. Microbiol. 27*, 317-346.

Güther, M.L.S., Lee, S., Tetley, L., Acosta-Serrano, A. and Ferguson, M.A.J. (2006). GPI-anchored proteins and free GPI glycolipids of procyclic form *Trypanosoma brucei* are nonessential for growth, are required for colonization of the tsetse fly, and are not the only components of the surface coat. *Mol. Biol. Cell 17*, 5265-5274.

Gutteridge, W.E. (1985). Existing chemotherapy and its limitations. *Br. Med. Bull. 41*, 162-168.

Haberkorn, A. and Gonnert, R. (1972). Animal experimental investigation into activity of Nifurtimox against *Trypanosoma cruzi*. *Arzneimittelforschung 22*, 1570-1582.

Hall, B.S., Bot, C. and Wilkinson, S.R. (2011). Nifurtimox activation by trypanosomal type I nitroreductases generates cytotoxic nitrile metabolites. *J. Biol. Chem. 286*, 13088-13095.

Hall, B.S., Wu, X.H., Hu, L.Q. and Wilkinson, S.R. (2010). Exploiting the drug-activating properties of a novel trypanosomal nitroreductase. *Antimicrob. Agents Chemother. 54*, 1193-1199.

Hecht, H.J., Erdmann, H., Park, H.J., Sprinzl, M. and Schmid, R.D. (1995). Crystal structure of NADH oxidase from *Thermus thermophilus*. *Nat. Struct. Biol. 2*, 1109-1114.

Henderson, G.B., Ulrich, P., Fairlamb, A.H., Rosenberg, I., Pereira, M., Sela, M. and Cerami, A. (1988). "Subversive" substrates for the enzyme trypanothione disulfide reductase: alternative approach to chemotherapy of Chagas' disease. *Proc. Natl. Acad. Sci. USA 85*, 5374-5378.

Hirumi, H. and Hirumi, K. (1989). Continuous cultivation of *Trypanosoma brucei* blood stream forms in a medium containing a low concentration of serum protein without feeder cell layers. *J. Parasitol. 75*, 985-989.

Hoffmann, K. (1972). Toxicological Investigations on Tolerability of Nifurtimox. *Arzneimittelforschung 22*, 1590-1603.

Janssens, P.G. and Demuynck, A. (1977). Clinical-trials with nifurtimox in African trypanosomiasis. *Ann. Soc. Belg. Med. Trop. 57*, 475-480.

Jansson, R., Malm, M., Roth, C. and Ashton, M. (2008). Enantioselective and nonlinear intestinal absorption of eflornithine in the rat. *Antimicrob. Agents Chemother. 52*, 2842-2848.

Jennings, F.W. (1993). Combination chemotherapy of CNS trypanosomiasis. *Acta Trop. 54*, 205-213.

Jennings, F.W. and Urquhart, G.M. (1983). The use of the 2 substituted 5-nitroimidazole, Fexinidazole (Hoe 239) in the treatment of chronic *T.brucei* infections in mice. *Z. Parasitenkd. -Parasitol. Res. 69*, 577-581.

Jones, D.C., Ariza, A., Chow, W.H., Oza, S.L. and Fairlamb, A.H. (2010a). Comparative structural, kinetic and inhibitor studies of *Trypanosoma brucei* trypanothione reductase with *T. cruzi*. *Mol. Biochem. Parasitol. 169*, 12-19.

Jones, D.C., Hallyburton, I., Stojanovski, L., Read, K.D., Frearson, J.A. and Fairlamb, A.H. (2010b). Identification of a kappa-opioid agonist as a potent and selective lead for drug development against human African trypanosomiasis. *Biochem. Pharmacol. 80*, 1478-1486.

Jordan, A.M. (1993). Tsetse-flies (Glossinidae). In *Medical Insects and Arachnids*, R.P. Lane and R.W. Crosskey, eds. (London: Chapman & Hall), pp. 333-388.

Kaminsky, R. and Zweygarth, E. (1991). The effect of verapamil alone and in combination with trypanocides on multidrug-resistant *Trypanosoma brucei brucei*. *Acta Trop. 49*, 215-225.

Keeffe, E.B. and Rossignol, J.-F. (2009). Treatment of chronic viral hepatitis with nitazoxanide and second generation thiazolides. *World J. Gastroenterol. 15*, 1805-1808.

Kelly, J.M., Taylor, M.C., Smith, K., Hunter, K.J. and Fairlamb, A.H. (1993). Phenotype of recombinant *Leishmania donovani* and *Trypanosoma cruzi* which overexpress trypanothione reductase: sensitivity towards agents that are thought to induce oxidative stress. *Eur. J. Biochem. 218*, 29-37.

Klokouzas, A., Shahi, S., Hladky, S.B., Barrand, M.A. and van Veen, H.W. (2003). ABC transporters and drug resistance in parasitic protozoa. *Int. J. Antimicrob. Agents 22*, 301-317.

Knox, R.J., Burke, P.J., Chen, S. and Kerr, D.J. (2003). CB 1954: From the Walker tumor to NQO2 and VDEPT. *Curr. Pharm. Des. 9*, 2091-2104.

Koder, R.L., Haynes, C.A., Rodgers, M.E., Rodgers, D.W. and Miller, A.F. (2002). Flavin thermodynamics explain the oxygen insensitivity of enteric nitroreductases. *Biochemistry 41*, 14197-14205.

Kubata, B.K., Kabututu, Z., Nozaki, T., Munday, C.J., Fukuzumi, S., Ohkubo, K., Lazarus, M., Maruyama, T., Martin, S.K., Duszenko, M. and Urade, Y. (2002). A key role for old yellow enzyme in the metabolism of drugs by *Trypanosoma cruzi*. *J. Exp. Med. 196*, 1241-1251.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680-685.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D.,

Gibson, T.J. and Higgins, D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics 23*, 2947-2948.

Likeufack, A.C.L., Brun, R., Fomena, A. and Truc, P. (2006). Comparison of the in vitro drug sensitivity of *Trypanosoma brucei gambiense* strains from West and Central Africa isolated in the periods 1960-1995 and 1999-2004. *Acta Trop. 100*, 11-16.

Linding, R., Russell, R.B., Neduva, V. and Gibson, T.J. (2003). GlobPlot: exploring protein sequences for globularity and disorder. *Nucleic Acids Res. 31*, 3701-3708.

Löfmark, S., Edlund, C. and Nord, C.E. (2010). Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clin. Infect. Dis. 50*, S16-S23.

Magnus, E., Vervoort, T. and Van Meirvenne, N. (1978). A card-agglutination test with stained trypanosomes (C.A.T.T.) for the serological diagnosis of *T.b. gambiense* trypanosomiasis. *Ann. Soc. Belg. Med. Trop. 58*, 169-176.

Maina, N., Maina, K.J., Maser, P. and Brun, R. (2007). Genotypic and phenotypic characterization of *Trypanosoma brucei gambiense* isolates from Ibba, South Sudan, an area of high melarsoprol treatment failure rate. *Acta Trop. 104*, 84-90.

Marr, J.J. and Docampo, R. (1986). Chemotherapy for Chagas' disease: a perspective of current therapy and considerations for future research. *Rev. Inf. Dis. 8*, 884-903.

Matthews, K.R. (2011). Controlling and coordinating development in vector-transmitted parasites. *Science 331*, 1149-1153.

Maya, J.D., Bollo, S., Nunez-Vergara, L.J., Squella, J.A., Repetto, Y., Morello, A., Périé, J. and Chauvière, G. (2003). *Trypanosoma cruzi*: effect and mode of action of nitroimidazole and nitrofuran derivatives. *Biochem. Pharmacol. 65*, 999-1006.

McCalla, D.R., Kaiser, C. and Green, M.H.L. (1978). Genetics of nitrofurazone resistance in *Escherichia coli*. *J. Bacteriol. 133*, 10-16.

Medenwald, H., Brandau, K. and Schlossm, K. (1972). Quantitative determination of Nifurtimox in body fluids of rat, dog and man. *Arzneimittelforschung 22*, 1613-1617.

Mehlert, A., Zitzmann, N., Richardson, J.M., Treumann, A. and Ferguson, M.A.J. (1998). The glycosylation of the variant surface glycoproteins and procyclic acidic repetitive proteins of *Trypanosoma brucei*. *Mol. Biochem. Parasitol. 91*, 145-152.

Mejia-Jaramillo, A.M., Fernandez, G.J., Palacio, L. and Triana-Chavez, O. (2011). Gene expression study using real-time PCR identifies an NTR gene as a major marker of resistance to benznidazole in *Trypanosoma cruzi*. *Parasites & Vectors 4*.

Miura, K. and Reckendorf, H.K. (1967). The nitrofurans. *Prog. Med. Chem. 5*, 320-381.

Moloo, S.K., Kabata, J.M. and Gitire, N.M. (2000). Study on the mechanical transmission by tsetse fly *Glossina morsitans centralis* of *Trypanosoma vivax*, *T. congolense* or *T. brucei brucei* to goats. *Acta Trop. 74*, 105-108.

Mukhopadhyay, R., Dey, S., Xu, N., Gage, D., Lightbody, J., Ouellette, M. and Rosen, B.P. (1996). Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. *Proc. Natl. Acad. Sci. USA 93*, 10383-10387.

Muller, J., Wastling, J., Sanderson, S., Muller, N. and Hemphill, A. (2007). A novel *Giardia lamblia* nitroreductase, GlNR1, interacts with nitazoxanide and other thiazolides. *Antimicrob. Agents Chemother. 51*, 1979-1986.

Murta, S.M.F., Krieger, M.A., Montenegro, L.R., Campos, F.F.M., Probst, C.M., Avila, A.R., Muto, N.H., de Oliveira, R.C., Nunes, L.R., Nirde, P., Bruna-Romero, O., Goldenberg, S. and Romanha, A.J. (2006). Deletion of copies of the gene encoding old yellow enzyme (TcOYE), a NAD(P)H flavin oxidoreductase, associates with in vitro-induced benznidazole resistance in *Trypanosoma cruzi*. *Mol. Biochem. Parasitol. 146*, 151-162.

Murta, S.M.F. and Romanha, A.J. (1998). *In vivo* selection of a population of *Trypanosoma cruzi* and clones resistant to benznidazole. *Parasitology 116*, 165-171.

Neal, R.A., van Bueren, J., McCoy, N.G. and Iwobi, M. (1989). Reversal of drug resistance in *Trypanosoma cruzi* and *Leishmania donovani* by verapamil. *Trans. R. Soc. Trop. Med. Hyg. 83*, 197-198.

O'Brien, J., Wilson, I., Orton, T. and Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem. 267*, 5421-5426.

Odiit, M., Kansiime, F. and Enyaru, J.C.K. (1997). Duration of symptoms and case fatality of sleeping sickness caused by *Trypanosoma brucei rhodesiense* in Tororo, Uganda. *East Afr. Med. J. 74*, 792-795.

Odiit, M., Shaw, A., Welburn, S.C., Fevre, E.M., Coleman, P.G. and McDermott, J.J. (2004). Assessing the patterns of health-seeking behaviour and awareness among sleeping-sickness patients in eastern Uganda. *Ann. Trop. Med. Parasitol. 98*, 339-348.

Oza, S.L., Ariyanayagam, M.R., Aitcheson, N. and Fairlamb, A.H. (2003). Properties of trypanothione synthetase from *Trypanosoma brucei*. *Mol. Biochem. Parasitol. 131*, 25-33.

Panigrahi, A.K., Ogata, Y., Zikova, A., Anupama, A., Dalley, R.A., Acestor, N., Myler, P.J. and Stuart, K.D. (2009). A comprehensive analysis of *Trypanosoma brucei* mitochondrial proteome. *Proteomics 9*, 434-450.

Parkinson, G.N., Skelly, J.V. and Neidle, S. (2000). Crystal structure of FMN-dependent nitroreductase from *Escherichia coli B*: A prodrug-activating enzyme. *J. Med. Chem. 43*, 3624-3631.

Patel, B., Thompson, J., Zhang, Y., Gitendra-Wickremasinghe, R., Steele, A. and Fielding, A. (2009). A phase I/II clinical trial in localized prostate cancer of an adenovirus expressing nitroreductase with CB1954. *Mol. Therapy 17*, 1292-1299.

Paulos, C., Paredes, J., Vasquez, I., Thambo, S., Arancibia, A. and GonzalezMartin, G. (1989). Pharmacokinetics of a nitrofuran compound, nifurtimox, in healthy volunteers. *Int. J. Clin. Pharmacol. 27*, 454-457.

Pays, E. (2006). The variant surface glycoprotein as a tool for adaptation in African trypanosomes. *Microbes. Infect. 8*, 930-937.

Pays, E. and Nolan, D.P. (1998). Expression and function of surface proteins in Trypanosoma brucei. *Mol. Biochem. Parasitol. 91*, 3-36.

Pepin, J. and Meda, H.A. (2001). The epidemiology and control of human African trypanosomiasis. *Adv. Parasitol. 49*, 71-132.

Pépin, J. and Milord, F. (1994). The treatment of human African trypanosomiasis. *Adv. Parasitol. 33*, 1-47.

Pépin, J., Milord, F., Meurice, F., Ethier, L., Loko, L. and Mpia, B. (1992). High-dose nifurtimox for arseno-resistant *Trypanosoma brucei gambiense* sleeping sickness: an open trial in central Zaire. *Trans. R. Soc. Trop. Med. Hyg. 86*, 254-256.

Picozzi, K., Fevre, E.M., Odiit, M., Carrington, M., Eisler, M.C., Maudlin, I. and Welburn, S.C. (2005). Sleeping sickness in Uganda: a thin line between two fatal diseases. *BMJ 331*, 1238-1241.

Poltera, A.A., Hochmann, A. and Lambert, P.H. (1981). *Trypanosoma brucei brucei* - the response to melarsoprol in mice with cerebral trypanosomiasis - an immunopathological study. *Clin. Exp. Immunol. 46*, 363-374.

Portal, P., Villamil, S.F., Alonso, G.D., De Vas, M.G., Flawia, M.M., Torres, H.N. and Paveto, C. (2008). Multiple NADPH-cytochrome P450 reductases from *Trypanosoma cruzi* - Suggested role on drug resistance. *Mol. Biochem. Parasitol. 160*, 42-51.

Priotto, G., Fogg, C., Balasegaram, M., Erphas, O., Louga, A., Checchi, F., Ghabri, S. and Piola, P. (2006). Three drug combinations for late-stage *Trypanosoma brucei gambiense* sleeping sickness: a randomized clinical trial in Uganda. *PLoS Clin. Trials 1*, e39.

Priotto, G., Kasparian, S., Mutombo, W., Ngouama, D., Ghorashian, S., Arnold, U., Ghabri, S., Baudin, E., Buard, V., Kazadi-Kyanza, S., Ilunga, M., Mutangala, W., Pohlig, G., Schmid, C., Karunakara, U., Torreele, E. and Kande, V. (2009). Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. *Lancet 374*, 56-64.

Privé, G.G. (2007). Detergents for the stabilization and crystallization of membrane proteins. *Methods 41*, 388-397.

Race, P.R., Lovering, A.L., Green, R.M., Ossor, A., White, S.A., Searle, P.F., Wrighton, C.J. and Hyde, E.I. (2005). Structural and mechanistic studies of *Escherichia coli* nitroreductase with the antibiotic nitrofurazone - Reversed binding orientations in different redox states of the enzyme. *J. Biol. Chem. 280*, 13256-13264.

Raether, W. and Hänel, H. (2003). Nitroheterocyclic drugs with broad spectrum activity. *Parasitol. Res. 90*, S19-S39.

Raether, W. and Seidenath, H. (1983). The activity of Fexinidazole (Hoe-239) against experimental infections with *Trypanosoma cruzi*, Trichomonads and *Entamoeba histolytica*. *Ann. Trop. Med. Parasitol. 77*, 13-26.

Rassi, A., Rassi, A. and Marin-Neto, J.A. (2010). Chagas disease. *Lancet 375*, 1388-1402.

Reischl, U., Gerdes, C., Motz, M. and Wolf, H. (1996). Expression and purification of an Epstein-Barr virus encoded 23-kDa protein and characterization of its immunological properties. *J. Virol. Methods 57*, 71-85.

Robays, J., Nyamowala, G., Sese, C., Betu Ku Mesu Kande, V., Lutumba, P., Van der Veken, W. and Boelaert, M. (2008). High failure rates of melarsoprol for sleeping sickness, Democratic Republic of Congo. *Emerg. Infect. Dis. 14*, 966-967.

Roldan, M.D., Perez-Reinado, E., Castillo, F. and Moreno-Vivian, C. (2008). Reduction of polynitroaromatic compounds: the bacterial nitroreductases. *FEMS Microbiol. Rev. 32*, 474-500.

Ross, R. and Thomson, D. (1910). A case of sleeping sickness showing regular periodical increase of the parasites disclosed. *BMJ 1*, 1544-1545.

Sambrook, J. and Russell, D.W. (2001). Molecular Cloning : A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Schmid, C., Nkunku, S., Merolle, A., Vounatsou, P. and Burri, C. (2004). Efficacy of 10-day melarsoprol schedule 2 years after treatment for late-stage gambiense sleeping sickness. *Lancet 364*, 789-790.

Shahi, S.K., Krauth-Siegel, R.L. and Clayton, C.E. (2002). Overexpression of the putative thiol conjugate transporter *Tb*MRPA causes melarsoprol resistance in *Trypanosoma brucei*. *Mol. Microbiol. 43*, 1129-1138.

Shim, H. and Fairlamb, A.H. (1988). Levels of polyamines, glutathione and glutathione-spermidine conjugates during growth of the insect trypanosomatid *Crithidia fasciculata*. *J. Gen. Microbiol. 134*, 807-817.

Sienkiewicz, N., Jaroslawski, S., Wyllie, S. and Fairlamb, A.H. (2008). Chemical and genetic validation of dihydrofolate reductase-thymidylate synthase as a drug target in African trypanosomes. *Mol. Microbiol. 69*, 520-533.

Simarro, P.P., Cecchi, G., Paone, M., Franco, J.R., Diarra, A., Ruiz, J.A., Fevre, E.M., Courtin, F., Mattioli, R.C. and Jannin, J.G. (2010). The Atlas of human African trypanosomiasis: a contribution to global mapping of neglected tropical diseases. *Int. J. Health Geogr. 9*.

Simarro, P.P., Diarra, A., Ruiz Postigo, J.A., Franco, J.R. and Jannin, J.G. (2011). The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000-2009: the way forward. *PLoS Negl. Trop. Dis. 5*, e1007.

Simarro, P.P., Jannin, J. and Cattand, P. (2008). Eliminating human African trypanosomiasis: Where do we stand and what comes next? *PLoS Med. 5*, 174-180.

Singh, R., Manjunatha, U., Boshoff, H.I.M., Ha, Y.H., Niyomrattanakit, P., Ledwidge, R., Dowd, C.S., Lee, I.Y., Kim, P., Zhang, L., Kang, S.H., Keller, T.H., Jiricek, J. and Barry, C.E.I. (2008). PA-824 kills nonreplicating *Mycobacterium tuberculosis* by intracellular NO release. *Science 322*, 1392-1395.

Soignet, S.L., Tong, W.P., Hirschfeld, S. and Warrell, R.P. (1999). Clinical study of an organic arsenical, melarsoprol, in patients with advanced leukemia. *Cancer Chemother. Pharmacol. 44*, 417-421.

Sokolova, A.Y., Wyllie, S., Patterson, S., Oza, S.L., Read, K.D. and Fairlamb, A.H. (2010). Cross-resistance to nitro drugs and implications for treatment of human African trypanosomiasis. *Antimicrob. Agents Chemother. 54*, 2893-2900.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel-electrophoresis. *J. Mol. Biol. 98*, 503-517.

Stoppini, L., Buchs, P.A., Brun, R., Muller, D., Duport, S., Parisi, L. and Seebeck, T. (2000). Infection of organotypic slice cultures from rat central nervous tissue with *Trypanosoma brucei brucei*. *Int. J. Med. Microbiol. 290*, 105-113.

Stuart, K.D., Brun, R., Croft, S.L., Fairlamb, A.H., Gurtler, R.E., McKerrow, J.H., Reed, S. and Tarleton, R.L. (2008). Kinetoplastids: related protozoan pathogens, different diseases. *J. Clin. Invest. 118*, 1301-1310.

Tang, M.H.Y., Helsby, N.A., Wilson, W.R. and Tingle, M.D. (2005). Aerobic 2- and 4-nitroreduction of CB 1954 by human liver. *Toxicology 216*, 129-139.

Terpe, K. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotechnol. 60*, 523-533.

Tobias, J.W., Shrader, T.E., Rocap, G. and Varshavsky, A. (1991). The N-End Rule in bacteria. *Science 254*, 1374-1377.

Torreele, E., Trunz, B.B., Tweats, D., Kaiser, M., Brun, R., Mazue, G., Bray, M.A. and Pecoul, B. (2010). Fexinidazole - a new oral nitroimidazole drug candidate entering clinical development for the treatment of sleeping sickness. *PLoS Negl. Trop. Dis. 4*, e923.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA 76*, 4350-4354.

Townson, S.M., Boreham, P.F.L., Upcroft, P. and Upcroft, J.A. (1994). Resistance to the Nitroheterocyclic Drugs. *Acta Trop. 56*, 173-194.

Tsuhako, M.H., Alves, M.J.M., Colli, W., Filardi, L.S., Brener, Z. and Augusto, O. (1991). Comparative studies of nifurtimox uptake and metabolism by drug-resistant and susceptible strains of *Trypanosoma cruzi*. *Comp. Biochem. Physiol. C, Comp. Pharmacol. Toxicol. 99*, 317-321.

Vanhamme, L. and Pays, E. (2004). The trypanosome lytic factor of human serum and the molecular basis of sleeping sickness. *Int. J. Parasitol. 34*, 887-898.

Vickerman, K. (1965). Polymorphism and mitochondrial activity in sleeping sickness trypanosomes. *Nature 208*, 762-766.

Vickerman, K. (1985). Developmental cycles and biology of pathogenic trypanosomes. *Br. Med. Bull. 41*, 105-114.

Vickers, T.J. and Fairlamb, A.H. (2004). Trypanothione *S*-transferase activity in a trypanosomatid ribosomal elongation factor 1B. *J. Biol. Chem. 279*, 27246-27256.

Vickers, T.J., Greig, N. and Fairlamb, A.H. (2004). A trypanothione-dependent glyoxalase I with a prokaryotic ancestry in *Leishmania major*. *Proc. Natl. Acad. Sci. USA 101*, 13186-13191.

Vincent, I.M., Creek, D., Watson, D.G., Kamleh, M.A., Woods, D.J., Wong, P.E., Burchmore, R.J.S. and Barrett, M.P. (2010). A molecular mechanism for eflornithine resistance in African trypanosomes. *PLoS Pathog. 6*, e1001204.

Viode, C., Bettache, N., Cenas, N., Krauth-Siegel, R.L., Chauviere, G., Bakalara, N. and Perie, J. (1999). Enzymatic reduction studies of nitroheterocycles. *Biochem. Pharmacol. 57*, 549-557.

Vogelstein, B. and Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci. USA 76*, 615-619.

Wegner, D.H.G. and Rohwedde, R.W. (1972a). Effect of Nifurtimox in acute Chagas' infection. *Arzneimittelforschung 22*, 1624-1635.

Wegner, D.H.G. and Rohwedde, R.W. (1972b). Experience with Nifurtimox in chronic Chagas' infection - preliminary report. *Arzneimittelforschung 22*, 1635-1642.

Welburn, S.C. and Maudlin, I. (1999). Tsetse-typanosome interactions: Rites of passage. *Parasitol. Today 15*, 399-403.

Welburn, S.C., Picozzi, K., Fevre, E.M., Coleman, P.G., Odiit, M., Carrington, M. and Maudlin, I. (2001). Identification of human-infective trypanosomes in animal reservoir of sleeping sickness in Uganda by means of serum-resistance-associated (SRA) gene. *Lancet 358*, 2017-2019.

WHO Expert Committee (1998). Control and surveillance of African trypanosomiasis. *WHO Tech. Rep. Ser. 881*, 1-113.

Wiegand, I., Hilpert, K. and Hancock, R.E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc. 3*, 163-175.

Wilkinson, S.R., Taylor, M.C., Horn, D., Kelly, J.M. and Cheeseman, I. (2008). A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. *Proc. Natl. Acad. Sci. USA 105*, 5022-5027.

Williamson, J. (1962). Chemotherapy and chemoprophylaxis of African Trypanosomiasis. *Exp. Parasitol. 12*, 274-367.

Williamson, J. (1970). Review of chemotherapeutic and chemoprophylactic agents. In *The African Trypanosomiases*, H.W. Mulligan, ed. (London: Allen and Unwin), pp. 125-221.

Winkelmann, E. and Raether, W. (1977). 1-Methyl-2-(phenyl-oxymethyl)-5-nitro-imidazoles and process for their manufacture. U.S. patent 4,042,705.

Winkelmann, E. and Raether, W. (1978). Chemotherapeutically Active Nitro-Compounds. 4. 5-Nitroimidazoles (Part III). *Arzneimittelforschung 28-1*, 739-749.

Wirtz, E., Leal, S., Ochatt, C. and Cross, G.A.M. (1999). A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. *Mol. Biochem. Parasitol. 99*, 89-101.

Wyllie, S., Oza, S.L., Patterson, S., Spinks, D., Thompson, S. and Fairlamb, A.H. (2009). Dissecting the essentiality of the bifunctional trypanothione synthetase-amidase in *Trypanosoma brucei* using chemical and genetic methods. *Mol. Microbiol. 74*, 529-540.

Yun, O., Priotto, G., Tong, J., Flevaud, L. and Chappuis, F. (2010). NECT Is Next: Implementing the New Drug Combination Therapy for *Trypanosoma brucei gambiense* Sleeping Sickness. *PLoS Negl. Trop. Dis. 4*.

Zenno, S., Koike, H., Tanokura, M. and Saigo, K. (1996). Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from *Escherichia coli*, similar in biochemical properties to FRase I, the major flavin reductase in *Vibrio fischeri*. *J. Biochem. 120*, 736-744.

Zhang, S.P., Zubay, G. and Goldman, E. (1991). Low-usage codons in *Escherichia coli*, yeast, fruit-fly and primates. *Gene 105*, 61-72.

Appendix

Sokolova, A.Y., Wyllie, S., Patterson, S., Oza, S.L., Read, K.D. and Fairlamb, A.H. (2010). Cross-resistance to nitro drugs and implications for treatment of human African trypanosomiasis. *Antimicrob. Agents Chemother. 54*, 2893-2900.